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(54) Title: USES OF CYTOKINES

(57) Abstract: The invention relates to a method of treatment or prophylaxis of avian pathogenic disease in a bird comprising administering to the bird one or more avian cytokine polypeptides sufficient to stimulate the immune response of the bird to an antigen. The avian cytokine polypeptides may be administered directly or via a nucleic acid molecule. The method may further comprise administration of an antigen administered directly or via a nucleic acid molecule. The invention also includes vaccines and gene constructs for carrying out the method.

TITLE OF THE INVENTION

Uses of cytokines

FIELD OF THE INVENTION

The present invention relates generally to cytokines and genetic sequences encoding same, and uses therefor. In particular, the present invention is directed to the use of recombinant cytokines as an immune response modulator in avian species and in particular the use of Chicken Myelomonocyte Growth Factor (cMGF). The present invention is particularly useful in the prophylactic and therapeutic treatment of birds against viral disease and in particular Marek's Disease and the causative agent thereof.

BACKGROUND OF THE INVENTION

Recent advances in the area of recombinant DNA technology has provided unforseen opportunities for research in the medical and veterinary fields. In particular, much work is being conducted in the field of cytokine research, since these molecules are capable of regulating the proliferation, differentiation and function of a variety of specialized cell types, especially cells involved in mediating immune responses. As a result, the administration of recombinant cytokines or regulation of cytokine function and/or synthesis is being employed in research into the treatment of a range of disease conditions in humans and animals. The present invention provides novel reagents and methods which utilise recombinant cytokine polypeptides and nucleotides encoding same, for the treatment of disease conditions in birds.

Chicken myelomonocyte growth factor (cMGF) is a 27 kDa glycoprotein that stimulates the proliferation avian myeloblasts and monocytes. The molecular sequence of cMGF is most closely related to IL6 and mammalian granulocyte colony stimulating factor, sharing 40 and 54% amino acid identity respectively. cMGF is produced from macrophage cell line HD11 in different forms generated from a 24-kDa polypeptide precursor by co- and post-translational acquisition of one or two N-linked

oligosaccharides and by O-linked glycosylation. In addition, a fraction of cMGF is modified by long chain, chondroitinase-sensitive, sulfated glycans. This modification is tunicamycin-sensitive, suggesting that the sulfated glycans are attached to N-linked rather than to O-linked oligosaccharides. (Leutz A, *et al.* J Biol Chem (1988) 263:3905)

In vitro expression of cMGF has been performed in eukaryotic expression systems such as COS (York J.J. *et al.* J Immunol (1996) 156:2991) and yeast (Johnston JA *et al.* Poultry Science (1997) 76:178). cMGF has also been produced from transformed Myeloid leukemic cells (Metz T, *et al.* EMBO J 1991;10(4):837). These eukaryotic expression systems produced protein capable of stimulating proliferation of bone marrow cells *in vitro.* Additionally, bacterially expressed cMGF induces proliferation of myoblasts *in vitro* Leutz A. *et al.* EMBO J (1989) 8:175.) However, *in ovo* injection of rcMGF was unable to demonstrate efficacy against early mortality associated with *E.Coli* (Johnston JA *et al.* (1997)). *In vivo* expression of cMGF via a recombinant fowlpox virus vector has been shown to induce increased numbers and activation of circulating monocytes. (York J.J. *et al* (1996)).

Chicken IL-2 was the first non-mammalian IL-2 cloned. (Sundick, R.S. and Gill-Dixon, C *J. Immunol.*, 1997, 159:720 - accession no AF000631). Recombinant chicken IL-2 produced in prokaryotic and eukaryotic expression systems induces proliferation of chicken splenocytes *in vitro.* Endogenous chicken IL-2 appears to occur *in vitro* as a monomer of about 14.2 kDa and is secreted within 4 h after ConA stimulation. (Stepaniak JA, Shuster JE, Hu W, Sundick RS.J Interferon Cytokine Res. 1999 May;19(5):515-26.). Chicken IL2 contains five repeats of the "instability" motif ATTTA in the 3'untranslated region in exon 4. It is a single-copy gene, with neither structural (amino acid) nor promoter sequence polymorphisms identified. A number of potential regulatory sequences similar to those found in mammals have been identified in the promoter. These include (5'-3') a composite NF-AT/ "AP-1" element, a CD28 response element, an AP-1 element, an NF-AT element, and the AP-1 part of an AP-1/octamer composite element: (Kaiser P, Mariani P Immunogenetics. 1999 Jan;49(1):26-35.) US patent 6,190,901.which is incorporated herein be reference

teaches genetic sequences encoding chicken IL-2 (although it refers incorrectly to IL-15).

Interleukin-2 and interleukin-15 are related cytokines that stimulate the activity and proliferation of T cells in mammals. Though IL-2 and IL-15 both interact with the β and γ chains of the IL-2 receptor, and may share some elements of tertiary structure, the two polypeptides are not homologous and represent distinct gene products. (Kanai T, Thomas EK, Yasutomi Y, Letvin NL.The Journal of Immunology, (1996), 157: 3681). The chicken IL-15 gene (Genbank accession number AF152927) is expressed in many tissues including spleen, intestine, and muscle and in established macrophage, T lymphoma and fibroblast cell lines. Chicken IL-15 cDNA contains an open reading frame of 143 amino acids with a single potential N-linked glycosylation site. The predicted molecular weight of the Chicken IL-15 is 16 kDa.

The encoded protein of chicken IL-18 consists of 198 amino acids and exhibits approximately 30% sequence identity to IL-18 of humans and various others mammals. Sequence comparisons reveals a putative caspase-1 cleavage site at aspartic acid 29 of the primary translation product, indicating that mature ChIL-18 might consist of 169 amino acids (gene bank accession no AJ277865). Bacterially expressed ChIL-18 in which the N-terminal 29 amino acids of the putative precursor molecule were replaced by a histidine tag induced the synthesis of interferon-gamma (IFN-gamma) in cultured primary chicken spleen cells, indicating that the recombinant protein is biologically active. Schneider, K., et. al. *J. Interferon Cytokine Res.*, (2000) 20:879 which is incorporated herein by reference, teaches cDNA cloning of biologically active chicken interleukin-18.

In mammals, interferons (IFN) represent a family of cytokines that share the capacity to inhibit viral replication and to exert effects on immune function. (Weissmann, C., and Weber, H. (1986). *Prog. Nucleic Acid Res. 33,* 251-300). The production of IFNγ in mammals is restricted to activated T cells and NK cells. IFNγ stimulates macrophages to produce reactive nitrogen intermediates such as nitric oxide, nitrate and nitrite (Fast, D.J.

et al (1993). *J. Interferon Res. 13,* 271-277; Huang, S. *et al* (1993). *Science 259,* 1742-1745). USSN 08/765,381, which is incorporated herein by way of reference, teaches genetic sequences encoding avian IFNγ from chicken reticuloendotheliosis virus (REV)-transformed spleen cell cultures (Lowenthal, J.W. *et al* (1995a) *In*: Adv.Avian Immunol.Res.(Eds. Davison T.F., *et al.*) Carfax, Oxford. pp179-186; Lowenthal, J.W., *et al* (1995b) *J. Interferon Cyt.Res.15,* 933-938). That specification further teaches the isolation of homologous IFNγ -encoding genetic sequences from species other than chickens, gene constructs and viral vectors comprising said genetic sequences, methods for the production of recombinant avian IFNγ using the said gene constructs and viral vectors, and methods of prophylaxis and treatment using the recombinant polypeptides.

Birds suffer from a variety of diseases that represent a considerable cost to the poultry industry, including diseases that are produced by bacteria and viruses, such as, for example, infectious bronchitis virus, avian infectious laryngeotracheitis virus, Newcastle disease virus, Marek's disease virus, chicken anaemia virus, avian influenza virus, infectious Bursal disease virus, avian leukosis virus, pneumovirosis virus, *E.coli, Salmonella* ssp., *Eimeria* ssp. and *Mycoplasma* ssp., amongst others.

Marek's disease is a considerable problem in the poultry industry. The disease is characterized by strong early immunosuppression and development of T-lymphomas. Marek's disease virus (MDV) is a alphaherpesvirus isolated from chickens. Three serotypes of virus have been identified : serotype 1 including strains with oncogenic potential, serotype 2 including non-oncogenic MDV strains and serotype 3 limited to an herpesvirus isolated from turkey (HVT) and not pathogenic for chickens. Resistance to disease can be acquired after vaccination. Attenuated strains from serotype 1 (eg Rispens), strains from serotype 2 (eg SB1) and HVT are used to vaccinate against oncogenic MDV. Although less than 100% effective, HVT is the major vaccine used for broilers.. It is often used in combination with SB1 as a bivalent vaccine to enhance efficacy. Rispens is an attenuated Serotype 1 strain, used mostly for breeder stock or layers, being more expensive than HVT and SB1. These vaccines have been used alone or in combination to improve vaccination efficacy (Geerlings HJ *et al.* Acta Virol

(1999) 43:198). The continuing appearance of new hypervirulent strains of MDV necessitate improving vaccination efficiency and the development of new prophylactic strategies.

Oncogenic MDV induces an initial phase of viral replication during the first week following infection, restricted to B-lymphocytes and to few T-lymphocytes. There is then a latency phase restricted to T-lymphocytes, the final target of the transformation process. T-lymphomas are mostly of the CD4+ CD8- phenotype. Very little is known about innate and acquired immune responses involved in the control of the disease. The majority of studies on immune responses occurring during Marek's disease have identified cell-mediated immunity, especially specific and non-specific cytotoxic responses, mediated by T-lymphocytes and NK cells respectively, as important in early protective mechanisms against viral replication.

Studies of Marek's disease virus have identified ICP4, pp38, Meq, gC, gB (and it's fragment gB-G) and gD genes(Schat KA and Xing Z Dev. Comp Immon (2000) 24:201) as important immunogens by *in vitro* CTL assays. gB and gD have been incorporated into plasmids (WO9803659) for use as polynucleotide vaccines. gB has also been incorporated into a fowlpox expression system and shown to induce protection comparable to HVT vaccines (Liu X *et al.* Acta Virol (1999) 43:201).

SUMMARY OF THE INVENTION

Bibliographic details of the publications referred to in this specification by author are collected at the end of the description.

Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification appear after the claims.

Throughout this specification and the claims that follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will

be understood to imply the inclusion of a stated element or integer or group of elements or integers, but not the exclusion of any other element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. the invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

One aspect of the present invention provides a method of treatment or prophylaxis of avian pathogenic disease in a bird, said method comprising administering thereto an effective amount of one or more avian cytokine polypeptides for a time and under conditions sufficient to maintain, stimulate or enhance the immune response of said bird to an antigen.

Another aspect provides a method of treatment or prophylaxis of avian pathogenic disease in a bird, said method comprising administering thereto an effective amount of one or more cytokine polypeptides for a time and under conditions sufficient to stimulate the immune response of said bird, wherein said cytokine polypeptide is selected from the group consisting of:

(a) a polypeptide having the amino acid sequence set forth in any one of SEQ ID NOs:2, 4, 6, 8 or 10;

(b) a polypeptide having the amino acid sequence set forth as the mature protein region of any one of SEQ ID NOs: 2, 4, 6, 8 or10;

(c) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence, wherein said nucleic acid molecule hybridizes under conditions of at least moderate stringency with a probe having a sequence

complementary to at least 50 contiguous nucleotides of SEQ ID NOs:1, 3, 5, 7 or 9;

(d) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence that is degenerate with a DNA molecule according to (c);

(e) a polypeptide comprising at least 10 contiguous amino acids of any one of SEQ ID NOs: 2, 4, 6, 8 or 10, wherein said polypeptide has immunomodulatory activity; and

(f) a homologue, analogue or derivative of any of the polypeptides of (a) to (e) wherein said polypeptide has immunomodulatory activity.

Because of the maintenance, stimulation or enhancement of the immune response of birds administered with the cytokine polypeptide, this aspect of the invention relates further to the enhancement and/or stimulation of an immune response to one or more antigens in a bird, wherein an immunomodulatingly effective amount of a cytokine polypeptide is optionally administered with, although not necessarily at the same time as, an antigen or pathogenic agent against which an immune response is desired. In such an embodiment of the invention, the administered cytokine polypeptide may act as an adjuvant, such as, for example, may be used in a vaccine composition.

The invention thus provides a vaccine composition for the treatment or prophylaxis of a bird against a pathogenic disease comprising an effective amount of one or more avian cytokine polypeptides or nucleic acid molecule encoding same and an immunomodulator or nucleic acid molecule encoding same.

Further the invention provides a vaccine composition for the prophylactic treatment of a bird against a pathogenic organism comprising an antigen in combination with an amount of one or more cytokine polypeptides selected from the group consisting of:

(a) a polypeptide having the amino acid sequence set forth in any one of SEQ ID NOs:2, 4, 6, 8 or 10;

(b) a polypeptide having the amino acid sequence set forth as the mature protein region of any one of SEQ ID NOs: 2, 4, 6, 8 or10;

(c) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence, wherein said nucleic acid molecule hybridizes under conditions of at least moderate stringency with a probe having a sequence complementary to at least 50 contiguous nucleotides of SEQ ID NOs:1, 3, 5, 7 or 9;

(d) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence that is degenerate with a DNA molecule according to (c);

(e) a polypeptide comprising at least 10 contiguous amino acids of any one of SEQ ID NOs: 2, 4, 6, 8 or 10, wherein said polypeptide has immunomodulatory activity; and

(f) a homologue, analogue or derivative of any of the polypeptides of (a) to (e) wherein said polypeptide has immunomodulatory activity.

In another aspect, the invention provides a gene construct comprising a first nucleotide sequence encoding a cytokine polypeptide and a second nucleotide sequence encoding an immunomodulator.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. shows blood smears obtained from chickens inoculated with fp/cMGF or fp/M3 vector seven days previously or left non-treated. Monocytes were labelled with 68.1 monoclonal anti-monocyte antibody and stained with goat anti-mouse IgG polyclonal antiserum labelled with fluorescein.

Figure 2. Qualitative RT-PCR amplification of mRNA of β-actin and iNOS, ChIFN-γ and K203 cytokines from B13/B13 chicken splenocytes 7 days following inoculation of fp/cMGF or fp/M3 vector. The data shown are the results for individual chickens. Thirty

amplification cycles were performed for ChIFN-γ and iNOS, 28 cycles for β-actin and 34 cycles for K203.

Figure 3. Semi-quantitative PCR from total blood for determination of MDV viremia. Two-week-old B13/B13 histocompatible chickens were inoculated with fp/cMGF or fp/M3 (10^5 PFU per chicken). Seven days later, chickens were challenged with RB-1B (10^3 PFU per chicken). Total blood DNA was extracted 21 days after RB-1B inoculation and viremia levels were determined using specific semi-quantitative PCR. Each bar represents a PCR result for one chicken. The chickens are in order of increasing PCR value within each group.

Figure 4. Effect of fp/cMGF and fp/M3 on survival of HVT-vaccinated chickens or unvaccinated chickens inoculated with RB-1B. Two-week-old B13/B13 chickens (12 chickens per group) were inoculated with fp/cMGF or fp/M3 vector (10^5 PFU per chicken) or left untreated. Vaccination with HVT (10^3 PFU per chicken) was performed 4 days after fp inoculation and RB-1B was inoculated 3 days later (i.e. 7 days after fp inoculation). All non-vaccinated chickens died by 5 weeks after RB-1B challenge. All remaining HVT-vaccinated chickens were sacrified 13 weeks after RB-1B challenge.

Fig 5. Effects of rcMGF on mortality of unvaccinated and HVT-vaccinated B^{13}/B^{13} chickens inoculated with RB-1B at 2 weeks of age. B13/B13 chickens (25 chickens per group) were treated with rcMGF (two daily injections of 50 μg per chicken) or left untreated. Vaccination with HVT (1000 PFU per chicken) was performed on the last day and RB-1B was inoculated 3 days later. All non-vaccinated chickens died by 6 weeks after RB-1B challenge. All remaining HVT-vaccinated chickens were sacrificed 10 weeks after RB-1B challenge.

Fig 6. Effects of rcMGF on mortality of unvaccinated and HVT-vaccinated B^{13}/B^{13} chickens inoculated with RB-1B at 3 days of age. B13/B13 chickens (30 chickens per group) were treated with rcMGF (50 μg per chicken) or left untreated. Vaccination with

HVT (1000 PFU per chicken) was performed the same day and RB-1B was inoculated 3 days later. All non-vaccinated chickens died by 26 days after RB-1B challenge. All remaining HVT-vaccinated chickens were sacrificed 30 days after RB-1B challenge.

DETAILED DESCRIPTION OF THE INVENTION

Livestock such as poultry, domestic birds and game birds are highly susceptible to infectious diseases, such as those transmitted by viruses, bacteria or Mycoplasma. Some of the more commercially important avian viral infectious agents include infectious bursal disease virus (IBDV), avian infectious bronchitis virus (IBV), avian infectious laryngeotracheitis virus (ILTV), Newcastle disease virus (NDV), Marek's Disease virus (MDV), chicken anemia virus (CAV) or avian influenza virus (AIV), avian leukosis virus, pneumovirosis virus amongst others and important pathogenic agents include *E.coli, Salmonella* ssp. or *Eimeria* ssp., amongst others.

The present invention provides an opportunity to maintain, stimulate or enhance the immune response of birds and in particular poultry, domestic birds or game birds, to an antigen by the administration of an avian cytokine, in particular cMGF, chIFNγ, chIL-2, chIL-15 and chIL-18 or a derivative thereof, either directly or via the expression of recombinant genetic sequences. This is of particular importance since most subunit and synthetic peptide vaccines are only weakly antigenic and means of enhancing the immune response to disease is generally beneficial. The administration of the cytokine may be alone, in combination with an antigen, other cytokines or as a fusion molecule. Administration may be via an direct DNA injection, attenuated virus, recombinant viral vector or bacterial vector or may be by administration of the cytokine by, for example, injection, aerosol or oral ingestion (e.g. in medicated foodstuff or water).

One aspect of the present invention provides a method of treatment or prophylaxis of avian pathogenic disease, said method comprising administering thereto an effective amount of a cytokine polypeptide for a time and under conditions sufficient to maintain,

stimulate or enhance the immmune response of said bird, wherein said cytokine polypeptide is selected from the group consisting of:

(a) a polypeptide having the amino acid sequence set forth in any one of SEQ ID NOs:2, 4, 6, 8 or 10;

(b) a polypeptide having the amino acid sequence set forth as the mature protein region of any one of SEQ ID NOs: 2, 4, 6, 8 or10;

(c) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence, wherein said nucleic acid molecule hybridizes under conditions of at least moderate stringency with a probe having a sequence complementary to at least 50 contiguous nucleotides of SEQ ID NOs:1, 3, 5, 7 or 9;

(d) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence that is degenerate with a DNA molecule according to (c);

(e) a polypeptide comprising at least 10 contiguous amino acids of any one of SEQ ID NOs: 2, 4, 6, 8 or 10, wherein said polypeptide has immunomodulatory activity; and

(f) a homologue, analogue or derivative of any of the polypeptides of (a) to (e) wherein said polypeptide has immunomodulatory activity.

By "prophylaxis" is meant the prevention of disease or severity of disease symptoms which are induced by infection with a viral or bacterial pathogen. Prophylaxis is usually brought about prior to exposure to said pathogen by immune stimulation, thus conferring immunity from infection, for example, as achieved by vaccination.

The term "avian" means a member of the class of vertebrates commonly referred to as birds. As used herein, the term "avian" includes both sexes and all developmental stages of poultry species, domestic birds and game birds selected from the list comprising chickens, turkeys, bantams, quails, guinea fowl, ducks, geese, ostriches, emus, pigeons, canaries, budgerigars, parrots and finches, amongst others.

The term cytokine is used generically for a diverse group of soluble proteins and peptides which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins may also mediate interactions between cells directly and regulate processes taking place in the extracellular environment.

Hereinafter the term "cytokine polypeptide" shall be taken to refer to a polypeptide molecule comprising at least one subunit of a biologically-active protein which possesses one or more of the characteristic biological features of a cytokine, in particular the ability to affect the functions of a cell which functions in the immune system of an animal as an immunomodulatory molecule in an avian species. Preferably it is capable of stimulating proliferation or differentiation of specific cell types; in particular the proliferation, activation or differentiation of lymphocytes, macrophages, mast cells, natural killer cells, granulocytes, inducing macrophages to secrete reactive nitrogen intermediates such as nitrite, nitrate or nitric oxide, amongst others or inducing cells to secrete cytokines.

The cytokine polypeptide includes cytokine polypeptides that have been modified chemically to extend the half-life or longevity of said polypeptide, such as, for example, by the addition of one or more protecting groups. Those skilled in the art will be aware of protecting groups other than the PEG substituents that may be used to modify the cytokine polypeptides of the invention. The present invention clearly extends to the use of chemical modifications to extend the half-life or longevity of said cytokine polypeptide.

Reference herein to "cytokine polypeptide" shall also be taken to include all possible fusion molecules between said cytokine polypeptide and another polypeptide, in particular an antigen or immunomodulatory molecule.

The cytokine polypeptide according to the present invention must possess functional and/or structural similarity to a cytokine, such as, for example, sufficient structural similarity so as to be immunologically cross-reactive with a cytokine, or to be at least 40% identical thereto at the amino acid sequence level as determined using any algorithm known by those skilled in the art.

In a preferred embodiment, the cytokine polypeptide is derived from a species of poultry selected from the list comprising chickens, ducks, geese, turkeys, bantams, quails or guinea fowl, amongst others. In a particularly preferred embodiment, the cytokine polypeptide is derived from chickens.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

Preferably the cytokine polypeptide is selected from a colony stimulating factor, interferon or interluekin. More preferably the cytokine is selected from myelomonocyte growth factor, interferon γ, IL-2 , IL-15 and IL-18 or subunits thereof. Even more preferably, the cytokines are avian or chicken cytokines. Most preferably the cytokines have the sequences of SEQ ID NOs: 2, 4, 6, 8 or 10 or a homologue, analogue or derivative thereof including any single or multiple amino acid substitutions, deletions and/or additions thereto .

The administration of cytokine polypeptides to avians provides a variety of useful outcomes. In particular, the administration of cytokines enhances growth of the subject, enhances proliferation, activation or differentiation of cells, including bone marrow cells, B cells, T cells, macrophage, monocytes. Consequently, the administration of a cytokine polypeptide to a subject may stimulate the immune response to inhibit infection or the progression of a diseased state, resulting in reduced symptoms such as weight loss and tumour growth, morbidity, mortality or pathogen load.

Remarkably, the cytokine polynucleotide has a potent booster effect which results in an amplification of the immune response and the acquisition of a long-lasting immunity.

As used herein, reference to an "immune response" includes reference to any specific or non-specific, humoral, cell mediated or innate response to an agent, such as, for example, a disease-promoting agent, a pathogen, an infectious agent, a tumour or a cancer-inducing agent, amongst others. An effective immune response will be able to suppress, inactivate, isolate, damage, reduce or remove the agent from the body, by a variety of means known to those skilled in the art. As used herein reference to "maintain, stimulate or enhance" the immune response shall include the initiation or stimulation of an inadequate, ineffective or absent immune response, as well as the alteration of the type or extent of immune response.

Some cytokines, such as IL-2, IL-15, IL-18 and IFNγ polarize the immune response toward Th1 cell development and stimulation of CTL activity thus directing enhancing a cell mediated immune response. In contrast, cytokines such as IL-4 promote the development of Th2 cells and increases production of antibodies, but suppresses CTL activity, favouring a humoral immune response. Thus, the immune responses to can be engineered by injection of one or more appropriate cytokine polypeptide or gene encoding a cytokine polypeptide to favor the formation of a suitable type of immune response, for example, either induction of CTL or neutralization antibodies as appropriate for a particular pathogen. Furthermore, the cytokines may enhance innate immunity.

Preferably, avian cytokines may induce macrophages to become activated, as measured by the increased expression of Class II molecules on their surfaces and/or the increased secretion of active nitrogen intermediates such as nitrites, thereby increasing the capacity of the immune system to destroy invading pathogens and to enhance the immune response thereto.

As used herein, the term "immunomodulatory molecule" shall be taken to refer to a composition of matter that is capable of altering any immune response of an animal to any antigen, such as, for example, a composition of matter that is a peptide, polypeptide, protein, vaccine, hapten, adjuvant, or organism, amongst others. Accordingly, an "immunomodulatory molecule" includes a peptide, polypeptide, protein, subunit vaccine, or killed organism that is capable of altering the immune response of an animal to an agent, such as, for example, a disease-promoting agent, an infectious agent, a tumour or a cancer-inducing agent, amongst others.

The present invention is particularly directed to a cytokine polypeptide which is at least capable of altering an immune response in a bird or alternatively a fusion molecule comprising same which is at least capable of altering an immune response in a bird or mammal to an antigen or infectious agent such as, but not limited to infectious bursal disease virus (IBDV), infectious bronchitis virus, avian infectious laryngeotracheitis virus, Newcastle disease virus, Marek's Disease virus, chicken anemia virus, avian influenza virus, avian leukosis virus, pneumovirosis virus, E.coli, Salmonella ssp., Eimeria ssp. or Mycoplasma ssp. amongst others, to alleviate symptoms associated therewith, in particular reduced growth performance.

As used herein, the terms "immunoresponsive-effective amount" and "immunomodulatingly effective amount" or similar, shall be taken to refer is an amount sufficient to effect immunomodulation in a target animal or to enhance the ability of the immune system to develop an effective immune response to an antigen or pathogenic organism and/or to enhance the immunogenicity of an antigen administered to said animal in a vaccine composition and/or to generally enhance the immunocompetence of the animal.

Recombinant cytokine polypeptides have been described, including those polypeptides derived from chickens and having the amino acid sequence set forth herein as SEQ ID NOs: 2, 4, 6, 8 and 10 and cDNA set forth in the present specification as SEQ ID

NOs:1, 3, 5, 7 and 9. However, it will be apparent to those skilled in the art that the present invention is readily performed using any avian derived cytokine polypeptide.

Other cytokine polypeptides derived from avians may be obtained by standard procedures known to those skilled in the art, such as, for example, by isolating the corresponding nucleic acid molecules using PCR or hybridisation approaches, and expressing the recombinant polypeptides therefrom. For example, homologous cMGF - encoding nucleic acid molecules may be obtained by hybridising avian nucleic acid under at least low stringency conditions to the nucleic acid molecule set forth in SEQ ID NO: 1, or to a complementary strand thereof. A "low stringency" is defined herein as being a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at 28 C, or equivalent. Those skilled in the art will be aware that the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash, and that the conditions for hybridisation and/or wash may vary depending upon the nature of the hybridisation membrane or the type of hybridisation probe used. Such conditions are well understood by one normally skilled in the art. For the purposes of clarification of the parameters affecting hybridisation between nucleic acid molecules, reference is found in Ausubel, F.M. *et al.* (1987) *In*: Current Protocols in Molecular Biology, Wiley Interscience (ISBN 047140338), which is herein incorporated by reference.

Particularly preferred homologues of the genes exemplified herein as SEQ ID NOs: 1, 3, 5, 7 or 9 may be obtained by hybridization under conditions of at least moderate stringency (i.e. 2xSSC buffer, 0.1% (w/v) SDS at 28 C, or equivalent) with a probe having a sequence complementary to at least 50 contiguous nucleotides of SEQ ID NOs:1, 3, 5, 7 or 9. Such homologues clearly include nucleotide sequences that are degenerate with SEQ ID NOs: 1, 3, 5, 7 or 9 (i.e. they encode the amino acid sequence set forth herein as SEQ ID NOs: 2, 4, 6, 8, or 10); and nucleic acid molecules that encode functional cMGF, IFNγ, IL-2, IL-15 or IL-18 polypeptides and comprise at least 10 contiguous amino acids of SEQ ID NOs: 2, 4, 6, 8 or 10.

In the case of PCR, one or more nucleic acid primer molecules of at least about 10 nucleotides in length derived from the genes of SEQ ID NOs: 1, 3, 5, 7 or 9 may be used to isolate such variant sequences.

Preferably, the administered polypeptide is a recombinant molecule. By "recombinant molecule" or "recombinant polypeptide" is meant a peptide, oligopeptide, polypeptide, protein or enzyme molecule that is produced by expressing non-endogenous nucleic acid encoding a cytokine polypeptide in a cell, tissue, organ or whole organism, such as, for example, the expression of foreign nucleic acid in a cell tissue, organ or whole organism that is different from the original cell, tissue, organ or organism from which said nucleic acid was derived, albeit not necessarily of a different species. Accordingly, a recombinant cytokine polypeptide may be produced by expressing a cytokine polypeptide-encoding nucleic acid in a chicken cell provided that said chicken cell is not the same cell from which said nucleic acid was originally derived (i.e. provided that the nucleic acid is non-endogenous), or alternatively, by expressing cytokine polypeptide -encoding nucleic acid in a non-chicken cell.

Several means may be employed to produce a recombinant polypeptide. Generally, a recombinant cytokine polypeptide will be produced following transfection of cells with the nucleic acid molecule encoding said polypeptide, wherein the introduced nucleic acid is maintained as an extrachromosomal element for a time and under conditions sufficient for expression to occur. In an alternative embodiment, the nucleic acid molecule may be expressed following its integration into the genome of a cell as an addition to the endogenous cellular complement of cytokine genes. Generally, to obtain expression, the introduced nucleic acid molecule contains a promoter sequence derived from the same or another gene, which regulates the expression of the cytokine gene sequence contained therein. Means for the introduction of nucleic acid to prokaryotic and eukaryotic cells will be well-known to those skilled in the art.

Preferably, the nucleic acid molecule that is used to produce a recombinant cytokine polypeptide comprises a sequence of nucleotides substantially the same as or

complementary to the nucleotide sequence set forth in SEQ ID NOs:1, 3, 5, 7 or 9 or a homologue, analogue or derivative thereof including any single or multiple nucleotide substitutions, deletions and/or additions thereto.

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as the nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

"Analogues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

"Derivatives" of a nucleotide sequence shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof. Generally, the nucleotide sequence is subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one

nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.

The present inventors have produced C-terminal addition variants of the cMGF cDNA sequence set forth in SEQ ID NO:1 of the present specification. In particular, the inventors have produced variants wherein additional amino acid residues in the form of a polyhistidine tag have been added to the C-terminal end of the cMGF polypeptide. The polypeptides were produced in E. coli cells and activity determined by expansion of monocytes in vivo. Accordingly, the present inventors have shown that despite the absence of post translational modifications, such as glycosylation, which would be expected to be important for the function of the glycoprotein, prokaryotic expression produces functionally active cMGF polypeptide.

The nucleic acid that is used to produce a recombinant cytokine polypeptide may comprise RNA or DNA or a combination thereof. Preferably, the nucleic acid is a gene that encodes a cytokine polypeptide. Reference herein to a "gene", is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5 - and 3 - untranslated sequences); and/or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) optionally comprising 5 - or 3 -untranslated sequences of the gene; and/or

(iii) synthetic or fusion nucleic acid molecules encoding all or part of a functional product.

Synthetic cytokine genes may be derived from a naturally-occurring cytokine gene by standard recombinant techniques. Generally, an cytokine gene may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions. Nucleotide insertional derivatives gene include 5 and 3 terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotides are introduced

into a predetermined site in the nucleotide sequence although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more nucleotides from the sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon. Alternatively, substituents are designed to alter one amino acid for another similar acting amino acid, or amino acid of like charge, polarity, or hydrophobicity.

To produce a recombinant cytokine polypeptide, the nucleic acid molecule encoding said polypeptide may be conveniently positioned within a gene construct, in operable connection with a suitable promoter sequence capable of conferring expression in the cell, tissue, organ or organism in which expression is desired.

By "gene construct" is meant a gene as hereinbefore defined operably connected to one or more non-encoding nucleotide sequences, such as, for example, a promoter sequence, an origin of replication or other sequence required for maintenance and/or replication in a cell, tissue, organ or whole organism. The term "gene construct" clearly includes within its scope isolated an isolated or synthetic nucleic acid molecule (or more particularly, an oligonucleotide) containing a cytokine polypeptide -encoding sequence that is operably connected to a promoter; and a plasmid vector, cosmid vector, bacteriophage vector, virus vector, or recombinant virus comprising said cytokine polypeptide -encoding sequence. As used herein, the term "vector" shall be taken to mean a nucleic acid molecule that is capable of being used to express a cytokine polypeptide in a cell, tissue, organ or organism (also known as an "expression vector") and preferably being maintained and/or replicated in a cell, tissue, organ or organism, and/or inserted into the chromosome of a cell, tissue, organ or organism.

Any number of expression vectors can be employed depending on whether expression is required in a eukaryotic or prokaryotic cell or a virus particle. Furthermore, it is well-

known in the art that the promoter sequence used in the expression vector will also vary depending upon the level of expression required and whether expression is intended to be constitutive or regulated.

DNA can be introduced to a subject for *in vivo* expression by viral or bacterial vectors or through uptake of 'naked' or complexed DNA. Preferred expression vectors are virus vectors. For expression in avian cells, it is particularly preferred to use a fowlpox vector (FPV), (as described in USSN 5,093,258 the contents of which are incorporated herein by way of reference) or a fowl adenovirus vector (FAV), (such as described in USSN 08/ 448, 617 and USSN 09/272, 032, the contents of which are incorporated herein by way of reference) or a Marek's disease viral vector or a Newcastle disease virus vector. In a particularly preferred embodiment, the vector comprises the right-hand end of FAV serotype 8 (hereinafter "FAV8"). The entire nucleotide sequence of the right-hand end of FAV8 is set forth herein as SEQ ID NO: 11. The entire nucleotide sequence of the FAV8 expression vector is also contained in GenBank Accession No. AF083975.

More particularly, the expression vector is a plasmid, which contains an 8.5 kilobase NheI fragment of the right-hand end of FAV8 wherein nucleic acid encoding cMGF in operable connection with a suitable promoter sequence may be substituted for the 1.3 kilobase SnaBI/SmaI FAV8 fragment of SEQ ID NO: 11, as is described in detail in (Johnson MA, Pooley C, Lowenthal JW. Delivery of avian cytokines by adenovirus vectors. Dev Comp Immunol 2000 Mar-Apr;24(2-3):343-54.).

More particularly the delivery of a cytokine polypeptide is via a vaccinal strain of fowlpox virus that has previously been shown to induce a strong and sustained systemic response to the cytokine in chickens (York JJ, Strom AD, Connick TE, McWaters PG, Boyle DB, Lowenthal JW. In vivo effects of chicken myelomonocytic growth factor: delivery via a viral vector.. J Immunol 1996 Apr 15;156(8):2991-7.).

For expression in plant cells, it is particularly preferred to use a tobacco mosaic virus (TMV) vector, however any plant virus-derived vector system may be used, such as, for

example, Gemini virus vectors, nanovirus vectors, and caulimovirus vectors, amongst others. Those skilled in the expression of proteins in plant cells will be aware of publicly available vectors within the scope of this description.

For expression in eukaryotic cells, the gene construct generally comprises, in addition to the nucleic acid molecule of the invention, a promoter and optionally other regulatory sequences designed to facilitate expression of said nucleic acid molecule. The promoter may be derived from a genomic clone encoding a cytokine polypeptide or, alternatively, it may be a heterologous promoter from another source. Promoter sequences suitable for expression of genes in eukaryotic cells are well-known in the art. In a preferred embodiment, the promoter is capable of expression in an avian cell.

Promoter sequences and culture conditions for cells or virus particles which produce high levels of expression are particularly preferred, and, according to this embodiment of the invention, it is particularly preferred for such promoter sequences to be capable of expressing a cytokine polypeptide for a time and at a level sufficient to produce an immunomodulatingly-effect amount of said polypeptide. Those skilled in the art will be aware that highly-active constitutive promoters will be particularly preferred. Such promoter sequences will be well-known to those skilled in the relevant art.

Particularly preferred promoters suitable for expression in animal cells, in particular avian cells, include the SV40 major later promoter (MLP), FAV major later promoter (MLP), cytomegalovirus immediate early promoter (CMVIE), or human adenovirus major late promoter, amongst others. Particularly preferred promoters for use in plant cells include the CaMV 35S or 19S promoter sequences, and nanovirus promoters, amongst others.

The prerequisite for producing intact polypeptides in E.coli is the use of a strong promoter with an effective ribosome binding site. Typical promoters suitable for expression in bacterial cells such as E. coli include, but are not limited to, the lacz promoter, temperature-sensitive L or R promoters, T7 promoter or the IPTG-inducible

tac promoter. A number of other vector systems for expressing the nucleic acid molecule of the invention in E.coli are well-known in the art and are described for example in Ausubel et al (1987) or Sambrook, J. *et al* (1989). *In*: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. It may also be the cytokine polypeptide- encoding gene's own promoter.

Numerous plasmid vectors with suitable promoter sequences for expression in bacteria and efficient ribosome binding sites have been described, such as for example, pKC30 (L: Shimatake and Rosenberg (1981) *Nature 292*, 128), pKK173-3 (tac: Amann and Brosius (1985) *Gene 40*, 183.), pET-3 (T7: Studier and Moffat (1986) *J. Mol. Biol. 189*, 113) or the pQE series of expression vectors (Qiagen, CA), amongst others.

There are numerous advantages of using DNA vectors for administration of cytokine polypeptide. The DNA encoded antigens are expressed as "pure" antigens in their native states and have undergone normal host cell modifications. DNA is easily and inexpensively manipulated, and is stable over a wide range of temperatures either as a dry product or in solution. This technology is valuable for the development of vaccines against practically any agent. The ability of directly injected DNA, that encodes a viral protein, to elicit a protective immune response has been demonstrated in numerous experimental systems. Vaccination through directly injecting DNA, that encodes a viral protein, to elicit a protective immune response produces both cell-mediated and humoral responses. This is analogous to results obtained with live viruses

A gene that encodes a cytokine polypeptide can be delivered by direct injection and provide long or short term exposure. DNA vaccines are typically comprised of naked or plasmid DNA molecules that encode a gene, such as an endogenous cytokine and may include antigen(s) derived from a pathogen or tumor cell. Following introduction into a vaccine, cells take up the DNA, where expression of the encoded cytokine polypeptide takes place.

Patent applications WO-A-90 11092, WO-A-92 19183, WO-A-94 21797 and WO-A-95 20660 have made use of the recently developed technique of polynucleotide vaccines. It is known that these vaccines use a plasmid capable of expressing, in the host cells, the antigen inserted into the plasmid. All the routes of administration have been proposed (intraperitoneal, intravenous, intramuscular, transcutaneous, intradermal, mucosal and the like). Various vaccination means can also be used, such as DNA deposited at the surface of gold particles and projected so as to penetrate into the animal's skin (Tang et al., Nature, 356, 152-154, 1992) and liquid jet injectors which make it possible to transfect at the same time the skin, the muscle, the fatty tissues and the mammary tissues (Furth et al., Analytical Biochemistry, 205, 365-368, 1992). (See also U.S. Pat. Nos. 5,846,946, 5,620,896, 5,643,578, 5,580,589, 5,589,466, 5,693,622, and 5,703,055; Science, 259:1745-49, 1993; Robinson et al., seminars in Immunology, 9:271-83, 1997; Luke et al., J. Infect. Dis. 175(1):91-97, 1997; Norman et al., Vaccine, 15(8):801-803, 1997; Bourne et al., The Journal of Infectious Disease, 173:800-7, 1996; and, note that generally a plasmid for a vaccine or immunological composition can comprise DNA encoding an antigen operatively linked to regulatory sequences which control expression or expression and secretion of the antigen from a host cell, e.g., a mammalian cell; for instance, from upstream to downstream, DNA for a promoter, DNA for a eukaryotic leader peptide for secretion, DNA for the antigen, and DNA encoding a terminator.)

DNA may be naked or formulated, for example, inside lipids or cationic liposomes.

In an alternative embodiment, the present invention extends to a gene construct comprising a nucleic acid molecule which encodes or is complementary to a nucleic acid molecule which encodes an avian cytokine polypeptide or a homologue, analogue or derivative thereof, wherein said polypeptide is a fusion polypeptide between a cytokine polypeptide and either a second cytokine or antigen. To produce such a fusion polypeptide, the nucleic acid molecule which encodes a first coding region comprising an avian cytokine polypeptide or a homologue, analogue or derivative thereof is cloned adjacent to a second coding region, optionally separated by a spacer nucleic acid

molecule such that the first coding region and the second coding region are in the same open reading frame, with no intervening stop codons between the two coding regions. When translated, the polypeptide thus produced comprises a fusion between the polypeptide products of the first and second coding regions. A gene construct which encodes a fusion polypeptide further comprises at least one start codon and one stop codon, capable of being recognised by the cell's translational machinery in which expression is intended. Methods for the production of a fusion polypeptide are well-known to those skilled in the art.

Recombinantly-produced cytokine polypeptide may be produced in a wide range of cell types. Examples of eukaryotic cells contemplated herein to be suitable for expression include avian, mammalian, yeast, insect, plant cells or cell lines such as COS, VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK) or MDCK cell lines. In particular, biologically-active cytokine polypeptide may be produced in *E.coli* cells, yeast cells, tobacco cells, COS cells, and chick kidney (CK) cells. Such cell lines are readily available to those skilled in the art.

Suitable prokaryotic cells include Corynebacterium, salmonella, Escherichia coli, Bacillus sp. and Pseudomonas sp, amongst others. Bacterial strains which are suitable for the present purpose are well-known in the relevant art (Ausubel et al, 1987; Sambrook et al, 1989).

The inventors cloned cytokine polypeptide encoding cDNA and produced recombinant gene constructs comprising same for the expression of recombinant cytokine polypeptides in viral and bacterial expression systems. The cMGF produced *in vitro* by a prokaryotic expression system was functional *in vivo* despite the absence of post translational modifications, such as glycosylation, which would be expected to be important for the function of the glycoprotein.

In a particularly preferred embodiments of the present invention, DNA is introduced to a subject for *in vivo* expression by viral mycoplasma or bacterial vectors or through

uptake of 'naked' or complexed DNA. Known viral vectors include Vaccinia, Fowlpox virus and Fowl Adenovirus. The cytokine polypeptide may also be delivered by a live delivery system such as using a bacterial expression system to express the cytokine polypeptide in bacteria which can be incorporated into gut flora. The inventive compositions described herein may utilize recombinant cMGF polypeptide, or cells infected with a recombinant viral vector expressing cMGF, or alternatively, recombinant viral vector expressing cMGF polypeptide, or nucleic acid encoding cMGF polypeptide. Particularly suitable viral vectors for the administration of cMGF polypeptide *in vivo* are fowl adenovirus (FAV) and fowlpox virus (FPV) into which the cMGF gene has been inserted in an expressible manner.

In a preferred embodiment, the invention provides administration of cytokine polypeptide to a bird selected from the group consisting of:

(i) healthy birds that are susceptible to infection.;
(ii) infected asymptomatic birds.; and
(iii) infected symptomatic birds.

In a further embodiment, the invention particularly relates to the prevention of tumour growth or mortality associated with Marek's Disease, wherein said cytokine polypeptide is administered to a bird selected from the group consisting of:

(i) healthy birds that are susceptible to infection by Marek's disease virus.;
(ii) asymptomatic birds infected with Marek's disease virus.; and
(iv) birds suffering from Marek's disease.

According to this embodiment, it is preferred to administer a composition comprising a cytokine polypeptide, preferably cMGF, for a time and under conditions sufficient to prevent tumour growth in said healthy or infected bird or to reduce tumour growth or prevent or delay mortality in said infected bird.

As used according to this aspect of the invention, the term "healthy bird" or similar shall be taken to mean a bird that exhibits no symptoms of a disease associated with or

known to cause tumour growth in birds. The term "healthy bird" clearly encompasses a bird that is susceptible to a disease associated with or known to cause tumour growth in birds, notwithstanding that it may not exhibit any symptoms associated therewith at the time of administration of said cytokine polypeptide.

As used according to this aspect of the invention, a "diseased bird" means a bird that has a disease, such as, for example, a disease associated with tumour growth in birds, or is infected with a causative agent thereof. Accordingly, a "diseased bird" includes a bird that exhibits one or more symptoms of a disease associated with, or known to cause, tumour growth in birds, including actual tumour growth. A "diseased bird" may also include a bird that exhibits no actual symptoms however has been diagnosed as carrying a causative agent of a disease associated with tumour growth in birds, particularly Marek's disease virus.

The immuno-responsiveness of birds administered with the subject cytokine polypeptide may be maintained, stimulated or enhanced by the optional administration of an immunomodulator, preferably a second cytokine polypeptide. In such an embodiment, the administration of a second cytokine may act to maintain, stimulate, suppress or repress the same or different aspects of the immune system, including the mitigation of a side effect of the first cytokine polypeptide.

Vaccination with cytokine-gene engineered tumor cells, fusion proteins between cytokines and tumor antigens, and their DNA can be used to induce or enhance immune response. For example, co-administration of cMGF and interleukin (IL)-2 may induce higher antibody titers and T-cell proliferation responses.

The invention thus provides a vaccine composition for the prophylactic treatment of a bird against a pathogenic organism comprising an antigen in combination with an amount of one or more cytokine polypeptides selected from the group consisting of:

a polypeptide having the amino acid sequence set forth in any one of SEQ ID NOs:2, 4, 6, 8 or 10;

(b) a polypeptide having the amino acid sequence set forth as the mature protein region of any one of SEQ ID NOs: 2, 4, 6, 8 or10;

(c) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence, wherein said nucleic acid molecule hybridizes under conditions of at least moderate stringency with a probe having a sequence complementary to at least 50 contiguous nucleotides of SEQ ID NOs:1, 3, 5, 7 or 9;

(d) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence that is degenerate with a DNA molecule according to (c);

(e) a polypeptide comprising at least 10 contiguous amino acids of any one of SEQ ID NOs: 2, 4, 6, 8 or 10, wherein said polypeptide has immunomodulatory activity; and

(f) a homologue, analogue or derivative of any of the polypeptides of (a) to (e) wherein said polypeptide has immunomodulatory activity.

In accordance with this embodiment of the invention, a cytokine polypeptide, is used to enhance the specific and non-specific responses to an antigen. For example, by increasing CTL responses, cell mediated or TH1 type response, persistence of effector cells and/or increased persistence, number and/or state of activation of monocytes and/or macrophage in immunised birds.

The term "antigen" as used herein means proteinaceous matter capable of recognition by antibody, B or T cell receptors, and the nucleotide sequence encoding that proteinaceous matter and includes natural and synthetic peptides, as well as live, killed and attenuated pathogens, subunit and DNA vaccines. An antigen may be in the form of a complete gene or fragments thereof for which the encoded peptide retains the capacity to be recognised.

It will be apparent from the disclosure herein that the administered cytokine polypeptide, with or without antigen and with or without a second cytokine, has

application as an adjuvant for vaccines, particularly for subunit or synthetic peptide vaccines produced by recombinant DNA technology. The adjuvant may also be administered in the traditional manner by injection of a combination of antigen and cytokine polypeptide, together or separately.

The antigen may be provided in the form of a conventional vaccine or plasmid construct. The vaccine may be monovalent or multivalent of the type in the prior art, in particular selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine.

Preferred antigens are the S, M and N antigens of infectious bronchitis virus, gB, gC and gD antigens of avian infectious laryngeotracheitis virus, HN and F antigens of Newcastle disease virus, gB and gB antigens of Marek's disease virus, C and NS1 antigens of chicken anaemia virus, HA, N and NP antigens of avian influenza virus, VP2 antigen of infectious Bursal disease virus, env and gag/pol antigens of avian leukosis virus, F and G antigens of pneumovirosis virus (referred to in WO 9803659 and incorporated herein by reference).

Preferred antigens are vaccines, including multivalent vaccines. Most preferred is a Marek's disease vaccine, for example, attenuated strains of serotype 1 (such as Rispens), strains of serotype 2 (such as SB1), or HVT. Each of these vaccines may be used alone or in combination to improve vaccination efficacy. Preferably HVT is used in combination with SB1 as a bivalent vaccine.

Valency in the present invention is understood to mean at least one antigen providing protection against the virus for the pathogen considered, it being possible for the valency to contain, as subvalency, one or more natural or modified genes from one or more strains of the pathogen considered.

The term "adjuvant" as used herein shall be taken to mean a substance that, when administered to an animal with a second substance or antigen, enhances the specific or

non-specific immune response to the second substance, for example, by enhancing the depot effect, activating macrophage or other antigen presenting cells or by biasing the immune response toward a Th1 or Th2 type response and as a result, increase antibody to T cell responses. An adjuvant is preferably administered in combination with a pharmaceutically-acceptable carrier, excipient or diluent.

According to one embodiment, there is contemplated a gene construct comprising:

(i) a first nucleotide sequence encoding a cytokine polypeptide, placed operably under the control of a first promoter sequence; and

(ii) a second nucleotide sequence defining an antigen against which immunisation is required, placed operably under the control of a second promoter sequence.

Preferably the construct also comprises a delivery vehicle comprising genetic sequences which facilitate replication of said gene construct in a delivery cell such as a bacterial, yeast, insect, a protozoan animal or a mammalian cell. The nucleotide sequences may be present in the same transcription unit or in two different units, in different plasmids or in one and the same plasmid, in the same or separate delivery vehicle.

Further, the gene construct may comprise more than one nucleotide sequence defining an antigen and/or more than one nucleotide sequence encoding a cytokine polypeptide

According to this embodiment, the delivery cell would not in normal use be harmful or pathogenic to the target animal. Conveniently, attenuated delivery vectors are employed. Particularly useful delivery vectors are attenuated viruses and recombinant viral mycoplasma and bacterial vectors. For example, an attenuated viral vector is used and the genetic sequence encoding CMGF polypeptide or a derivative thereof is cloned into the viral sequence and the recombinant virus used to infect target animals. The recombinant virus causes infection and replicates in the animal cells resulting in production of the recombinant cytokine. The infecting recombinant virus may subsequently be eliminated after production of an immunomodulatingly effective

amount of the recombinant cytokine. A similar protocol is adopted with live bacterial carriers. Alternatively, a non-replicating, non-infectious viral vector may be used. A non-replicating viral vector provides a means of introducing a genetic sequence which is transiently capable of expression of the desired cytokine because the non-replicating viral vector is not capable of cell-to-cell transmission.

In performing the invention, it will be apparent to those skilled in the art that the methods discussed for the expression of cytokine polypeptides in various cell type, including the use of gene constructs and expression vector systems therefor, and subsequent administration of cytokine polypeptides to birds, are equally applicable to the purpose of expression of antigen.

The cytokine polypeptide of the present invention may be administered throughout the life cycle of a bird for which treatment or prophylaxis is indicated. The developmental stage of the bird during which treatment or prophylaxis is most effective will vary depending upon the nature of the pathogen against which protection is sought, including its mode of transmission and period of highest infectivity. By "period of highest infectivity" is meant the developmental stage of the host during which it is most vulnerable to attack by a particular pathogen and/or during which there is a greater probability of incurring livestock losses or reduced productivity as a result of the pathogen infection. The parameters affecting optimum developmental stages of animals for administration of the subject cytokines will be well-known to those skilled in the art.

Newly hatched chickens have a relatively immature immune system and during the first few days post hatching T cells may be unresponsive to immune stimulation . It may be possible to overcome this problem by administering cytokines during or before this period thereby increasing immune responsiveness and reducing susceptibility to pathogens. Preferably the compositions of the present invention are administered *in ovo*. *In ovo* administration techniques that replace the injection of very young hatched chicks can increase the efficiency of administration and reduce the stress on young chicks caused by injection. Many methods of adding a beneficial material to avian eggs utilize

the injection of fluids by syringe. One traditional method has been syringe injection of eggs by hand. A number of automatic egg injection devices have also been developed. These include U.S. Pat. No. 5,056,464 to Lewis; U.S. Pat. Nos. 4,903,635 and 4,681,063 to Hebrank; U.S. Pat. No. 5,136,979 to Paul, et al.; and U.S. Pat. Nos. 4,040,388, 4,469,047, and 4,593,646 to Miller, which are incorporated herein by reference for their disclosure of egg injection equipment and methods.

The cytokine polypeptide of the invention or isolated nucleic acid molecule which encodes or is complementary to a nucleic acid molecule which encodes said cytokine polypeptide or, alternatively, a vector comprising a gene construct capable of expressing said cytokine in vivo or in ovo, for example a live recombinant viral vector, mycoplasma vector, live recombinant bacterial vector may be administered by any means including for example, by injection either in ovo or post-hatching, by injection such as intra-peritoneal, intra-dermal, intra-muscular, intra-ocular, intra-venous, intra-nasal, sub-cutaneous or other injection means, by ingestion as a medicated foodstuff or drinking water, by aerosol spray, where an effective amount is provided to the bird.

The efficiency of presentation to the immune system varies according to the tissues. In particular, the mucous membranes of the respiratory tree serve as barrier to the and are associated with lymphoid tissues which support local immunity. In addition, the administration by contact with the mucous membranes, in particular the buccal mucous membrane, the pharyngeal mucous membrane and the mucous membrane of the bronchial region, is certainly of interest for mass vaccination. Consequently, the mucosal routes of administration are a preferred mode of administration for the invention, using in particular neubilization or spray or drinking water.

The present invention clearly extends to compositions for use in performing the inventive methods supra, such as, for example, those compositions of cytokine polypeptides or nucleotides encoding such in combination with one or more suitable carriers and/or excipients and/or diluents, in particular those carriers and/or excipients and/or diluents suitable for veterinary use. The present invention also extends to include

the administration of a cytokine polypeptide or nucleotides encoding such and the optional second cytokine and the optional antigen at one or more time points and in one or more doses. Administration of the cytokine polypeptide may be concurrent, followed or preceded by administration of a conventional vaccine.

The present invention clearly extends to a veterinary pharmaceutical composition for use in poultry, domestic bird or game birds such as to enhance the immune system or accelerate its maturation or improve its immunocompetence or to facilitate immunomodulation in said birds, said composition comprising a recombinant cytokine polypeptide or a fusion molecule, in combination with an antigen or pathogenic organism against which it is desired to obtain immuno-protection, and optionally one or more carriers and/or diluents acceptable for veterinary use.

In an alternative embodiment, the present invention clearly contemplates the use of genetic vaccines and pharmaceutical compositions, wherein nucleic acid encoding the proteinaceous components is administered to the bird for expression therein. Wherein the composition comprises genetic material such as nucleic acid, it is administered "naked" or as part of a viral vector, bacterial vector or as a nucleic acid molecule encoded by a gene construct present in a delivery system such as a virus, yeast, bacterium, protozoan, insect, avian or mammalian cell. The expression of such a delivery system in a target animal will enable delivery of the recombinant avian cytokine.

The bird to be treated and the cytokine in the composition might be "homologous" in the sense that both are of the same species, or may be "heterologous" where the avian cytokine is effective in another bird species than the species from which it has been derived. The compositions may also contain other active molecules such as antibiotics or antigen molecules. Combinations of cytokine molecules with antigen molecules may increase the efficacy of the compositions.

The active ingredient(s) of the pharmaceutical composition is/are contemplated to exhibit excellent activity in stimulating, enhancing or otherwise facilitating an immune response in an animal species and in particular a poultry, domestic bird or game bird when administered in an amount which depends on the particular case. The variation depends, for example, on the cytokine and, in some cases, the antigen involved in stimulating the immune response. For example, from about 0.5 μg to about 20 mg of a particular cytokine which may be combined with other cytokines, per kilogram of body weight per day may be required. More preferably the dose is 5 to 500 mg of cytokine per day. Dosage regimen may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered in one or more of daily, weekly or monthly or in other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

Specific formulations such as liposomes, cationic lipids, may also be used for the manufacture of the vaccines.

The subject of the invention is also the method of administration comprising making a first administration of antigen and subsequently a booster with cytokine polynucleotide or nucleotides encoding such. More preferably, the cytokine polypeptide is administered first, and the antigen subsequently.

In a preferred embodiment of the invention, there is administered in a first instance, to the animal, the cytokine polypeptide or nucleotides encoding such, and after a period preferably of 1 day to 2 weeks, an effective dose of a conventional vaccine, especially inactivated, live, attenuated or recombinant, type, or alternatively a subunit vaccine is administered.

The active compounds may also be administered in dispersions prepared in glycerol, liquid polyethylene glycols, and/or mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for parenteral administration include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, antibiotics, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example. Advances in slow-release technology allow for the administration of pharmaceutically active substances over a prolonged period of time and include the use of emulsions and coatings.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient(s) into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The latter is particularly contemplated as far as the present invention extends to multivalent vaccines or multi-component cytokine molecules.

The present invention is further described by reference to the following non-limiting Examples:

Two-week-old White Leghorn chickens homozygous for the B13 major histocompatibility and born and raised at INRA (PAP, 37380 Nouzilly, France) under specific pathogen free (SPF) conditions were used for the experiments.

All cell lines and vectors referred to herein were publicly available or fully-described in York JJ *et al.*, which are incorporated herein by way of reference.

Example I

FPV vaccine strain vector (Fp/M3) and recombinant Fp/cMGF (York JJ *et al.* 1996) were propagated on monolayers of primary chicken embryo fibroblasts.

18 histocompatible B13/B13 chickens were inoculated intramuscularly with 10^5 PFU Fp/cMGF at two weeks of age, 18 were inoculated with the control Fp/M3 vector and 18 were left as non-inoculated control. The number of blood monocyte and nitric oxide (NO2- + NO3-) levels was measured in serum 3, 7 and 10 days later and spleen cytokine mRNA expression was measured 7 days after Fp/cMGF or Fp/M3 inoculation .

Fluorescence assay on blood smears

Blood smears were fixed with 75% ethanol, 25% acetone. Smears were then stained with monocyte-specofic 68.1 monoclonal antibody (Jeurissen SH, Janse EM, Koch G, de Boer GF. The monoclonal antibody CVI-ChNL-68.1 recognizes cells of the monocyte- macrophage lineage in chickens. Dev Comp Immunol 1988;12:855-64) at dilution 1 :10 and staining was revealed with secondary FITC-labelled goat anti-mouse polyclonal antibody (Jackson lab,). Sections were examined using an Olympus microscope with excitation/barrier filters for FITC.

Inoculation of fp/cMGF (10^5 PFU per chicken) quickly induced an increase in number of blood monocytes , identified by the specific monoclonal antibody 68.1, compared to control chickens inoculated with fp/M3 vector (FIG. 1). As early as 3 days post inoculation the number of blood monocytes was increased 2-fold and this significant increase persisted at this level for 7 days post inoculation.

Assay of nitric oxide (NO_2^- + NO_3^-) level in serum

The effects of fp/cMGF and fp/M3 were further tested on NO production (Table 1). Serum nitrate reflects systemic NO production. The sum of NO_2^- + NO_3^- has been confirmed to be a good indicator of NO production (Stuehr DJ, Marletta MA. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc Natl Acad Sci USA 1985;82:7738-42). The method described by Hegesh E, Shiloah J. (Blood nitrates and infantile methemoglobinemia. Clin Chim Acta 1982;125:107-15) was used with slight modifications.

The samples were protein precipitated (0.05 ml 30% zinc sulfate per ml) before analysis. Then 0.5 ml of the sample was incubated for 24 hours under agitation with granulated cadmium (0.6g) prewashed with distilled water, hydrochloric acid (0.1 mol), distilled water and ammonium hydroxide buffer (0.1 mol, pH 9.6). NO_3^- is reduced to NO_2^- by the cadmium. Fifty microliters of each sample was then collected and the quantity of nitrite

was measured using the Griess reaction by adding 50 µl of a freshly prepared mixture (50/50) of 1% sulfanilamide (Sigma) in 1.2 N hydrochloric acid and 0.3% N-1 naphthylethylenediamide dihydrochloride (Sigma) in a 96-well flat bottomed plate. Absorbance at 540 nm was determined after 10-min incubation in the dark. Nitrite concentrations were calculated by reference to a calibration curve prepared using standard solutions of sodium nitrite (starting at 200 µM) (Prolabo, Fontenay-sous-bois, France).

Statistical analysis

For nitrate levels, data were expressed as Means (+/- SEM). Variations between study group means were tested using the Kruskal-Wallis nonparametric ANOVA test (Simstat version 3.5b). Thereafter, differences between groups were examined with the Mann-Whitney test. Survival curves were compared at different time points using FISHER's EXACT test (Epi info version 5.01b).

Three, seven or ten days after inoculation, serum was taken before and after intravenous stimulation with LPS (50µg per chicken) to measure nitric oxide (NO2-+NO3-) level. Blood samples were obtained by cardiac puncture, allowed to clot for 4 hours and centrifuged for 10 mn at 3000g. The serum aliquots were stored at -20°C. The samples were protein precipitated (30% zinc sulfate, 0.05 ml per ml) before analysis. Then 0.5 ml of the sample was incubated for 24 hours under agitation with granulated cadmium (0.6g) prewashed with distilled water, hydrochloric acid (0.1 mol), distilled water and ammonium hydroxide buffer (0.1 mol, pH 9.6). NO_3^- is reduced to NO_2^- by the cadmium. Fifty microliters of each sample was then collected and the quantity of nitrite was measured using Griess reaction.

Table 1.
Effect of fp/cMGF on spontaneous and inducible nitric oxide (NO2-+NO3-) production in serum.

Days p.i	Treatment	Control	fp/M3	fp/cMGF
		NO2-+NO3- (μM)		
3	0	3.58 (1.1)	4.4 (1.3)	9.2 (1.2)**
	LPS 50μg	132.1 (30.6)	168.4 (6.4)	309.9 (11.1)**
7	0	6.2 (3.1)	5.1 (2.6)	11.3 (2.8)*
	LPS 50μg	133.4 (21.9)	98.1 (6.3)	257.7 (40.6)**
10	0	8.3 (1.3)	4.2 (1.3)	3.6 (0.9)*
	LPS 50μg	166.3 (18.8)	196.2 (58.2)	119.1 (30.8)

Results are expressed as mean values (±SEM) for 5 chickens per group per day. Significant difference was indicated for fp-inoculated chickens by comparison with control chickens *$p \leq 0.05$; **$p \leq 0.01$

Inoculation of fp/cMGF induced a 2-fold increase in serum nitrate production in the blood compared to non-infected controls as soon as 3 days post inoculation. This increase was still observed on day 7 post inoculation, but was no longer present 10 days after the inoculation. In addition, an enhanced response to macrophage activating stimuli such as LPS, was strongly exacerbated from 3 to 7 days following inoculation, resulting in a 2-fold increase in nitrate production in serum. No such enhancing effect on nitrate production in the serum was noted with fp/M3 vector alone. Thus seven days after fp inoculation was chosen for the MDV challenge when serum NO levels was maximum.

Detection of cytokine mRNA by RT-PCR

Spleens were removed aseptically. Single-cell suspensions in 1.1X PBS pH 7.4 (Gibco) were prepared by gently teasing the organ on a steel sieve. Nucleated erythrocytes were eliminated by centrifugation at 400g. Isolated splenocytes were used to study cytokine gene expression.

Splenocytes were homogenized in 1 ml of RNAble solution (Eurobio laboratory,) and total RNA was suspended in RNAse free sterile water. The amount and quality of RNA were dctermined by spectrophotometry and analyzed by agarose gel electrophoresis. A reverse transcriptase procedure was performed to determine relative quantities of mRNA for chicken IFN-γ , NOSi, K206 and β-actin as previously described with a few modifications. Reverse transcription of RNA was performed in 25 µl final volume containing 0.5 µg random oligo dT and 2.5 µg total RNA. The RT reaction was incubated for five minutes at 65°C and were then added 20 mM of each dNTP, 1X RT buffer (50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 8 mM DTT), 4 U RNAse Inhibitor, 200 U M-MLV Reverse transcriptase (Promega, France). The reaction was followed by one hour at 37°C, and completed with five minutes at 85°C to denature the M-MLV reverse transcriptase, cooled on ice for five minutes, and stored at –20°C. The primer sequences for the PCR reactions are listed in TABLE 2. PCR reaction conditions were previously defined for each pair cytokine-primer pair. The PCR reaction mixture contained 1) 1 mM of each dNTP, 2) 1X PCR buffer 50 mMKCl, 10 mM Tris-HCl, 3) 1 mM $MgCl_2$, 4) 12 pm of each sense and anti-sense primers 5) 2 µl cDNA 6) Taq polymerase (Promega,). After an initial denaturation step for 4 min at 92°C, followed by 1 min at 72°C, temperature cycling was initiated at 1) 92°C for 4 min, 2) 61°C for 1 min 3) 72°C for 1 min. Samples were stored at 4°C until use. An aliquot of each reaction mixture was subjected to electrophoresis in 2% agarose gels.

Table 2.

Primers used for RT-PCR analysis of chicken mRNAs.

Target mRNA	Accession number	5' Primer	3' Primer	Size of PCR Products (bp)
β-Actin	L08165	5'-CATCACCATTGGCAATGAGAGG-3'	5'-GCAAGCAGGAGTACGATGAATC-3'	353
IFNγ	U07868	5'-GATGACTTCCAGACTTACAAC-3'	5'-AGCAATTGCATCTCCTCTGAGACTG-3'	485
iNOS	U46504	5'-AGGCCAAACATCCTGGAGGTC-3'	5'-TCATAGAGACGCTGCTGCCAG-3'	371
K203	Y18692	5'-ATGAAGCTCTCTGCAGTTGTTCT-3'	5'-TCAGTCCCGCTTGACGCTCTG-3'	269

Three chickens per group were tested individually for iNOS, ChIFN-γ and K203 gene expression in the spleen. To allow comparison, the quantity of cDNA analyzed was adjusted for each chicken to give comparative amounts of β-actin gene expression. Spleen leucocytes isolated from the spleen of control B13/B13 chickens exhibited iNOS expression at three weeks of age in the experimental conditions used (FIG. 2). However no expression of ChIFN-γ or K203 genes was detectable in these animals. Inoculation of fp/M3 induced weak ChIFN-γ gene expression one of three chickens and no detectable expression in the other two. In this group, K203 expression was high in one chicken and low in the other two. Fp/M3-inoculated chickens showed the same level of iNOS gene expression as the control birds. In contrast, fp/cMGF inoculation induced strong ChIFN-γ, K203 and iNOS gene expression in spleen of all three tested chickens tested seven days post inoculation.

Example II

10 B13/B13 chickens were inoculated at two weeks of age with Fp/cMGF and 10 chickens with Fp/M3 as for Experiment I:

5 chickens from each group were then challenged with RB-1B 7 days later to measure RB-1B viremia in blood leucocytes in the fibroblast assay. Five control chickens were inoculated only with RB-1B. 10^3 PFU were inoculated intramuscularly per chicken. Blood leucocytes were isolated on MSL 21 days after RB-1B inoculation and viremia level was determined from RB-1B PFU recovery on chicken embryo fibroblasts.

The hypervirulent strain of MDV, RB-1B, was maintained by successive passages on SPF outbred chickens.

In vitro fibroblast assay for determination of MDV viremia

Eggs from a specific pathogen-free (SPF) flock (outbred White Leghorn) were the source of 11-day-old embryos needed for preparation of fibroblast cultures. Primary chicken embryo fibroblast cultures (CEF I) were prepared by standard methods in medium 199 (7% FCS) and seeded (10^6/well) in 6-well culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ., USA). CEF were grown to confluence in 6-well plates for 2 days and then infected with blood leucocytes ($2x10^6$ cells/well). Cells were then washed two days later. Blood leukocytes were obtained after centrifugation of heparinised blood (50 IU/ml) on MSL 1077 (Eurobio) for 20 mn at room temperature. RB-1B replication in CEF cells was evaluated by counting the number of PFU per well under the microscope after 7 days of culture.

TABLE 3.
Effect of fp/M3 or fp/cMGF on viremia after RB-1B infection

Groups	PFU/$2x10^6$ blood leucocytes
Uninfected CEF	0
CEF (fp/M3)	0
CEF (fp/cMGF)	0
CEF (RB-1B)	251 (38)
CEF (fp/M3 + RB-1B)	200 (41)
CEF (fp/cMGF + RB-1B)	94 (37)***

Values are given as means (± SEM) (5 chickens per group). Statistical significance was compared to CEF (RB-1B) ***$p \leq 0.001$.

fp/cMGF treatment inhibits *in vivo* MDV replication.

RB-1B viremia was evaluated three weeks following inoculation by two methods. Since MDV is characterized by strong cell (typically T lymphocyte) association and is not found in the supernatant or serum blood leucocytes were first isolated and cultured on chicken embryo fibroblasts. The number of PFU was measured one week after contact of blood leucocytes with fibroblasts (TABLE 3). No virus was observed in blood leucocytes from control chickens inoculated with both types of fowlpox but not challenged with RB-1B. Treatment with fp/cMGF resulted three weeks after RB-1B challenge in a significant 2.7-fold reduction in the RB-1B PFU recovered from blood leucocytes. In contrast, the reduction in RB-1B PFU by fp/M3 was not significant.

Example III

12 non-infected B13/B13 chickens were left as controls and 36 chickens were inoculated with RB-1B (as described above) divided in three groups of 12 : one group was inoculated 7 days previously with Fp/cMGF, one group with Fp/M3 vector and the third group was not treated. In these chickens, spontaneous and LPS-inducible nitric oxide (NO2- + NO3-) levels in serum was measured one and 3 weeks after MDV challenge as described above.

TABLE 4.
Effect of RB-1B infection on spontaneous and inducible nitric oxide (NO2-+NO3-) production in serum

Weeks p.i.	Treatment	Control	RB-1B	fp/M3 +RB-1B	fp/cMGF+RB-1B
		NO2- +NO3- (μM)			
1	0	11.4 (2.1)	41.5 (15.0)	31.2 (3.9)	25.2 (10.3)
	LPS 50μg	134.8 (28.5)	175.5 (37.7)	90.3 (52.9)	154.3 (74.4)
3	0	17.5 (0.9)	18.9 (5.8)	15.88 (3.7)	16.1 (4.0)
	LPS 50μg	156.6 (15.2)	402.2 (33.7)	446.5 (151.4)	371.5 (103.1)

Results are expressed as mean values (±SEM) for 5 chickens per group per day. Significant difference was indicated for fp-inoculated chickens by comparison with control chickens *p≤0.05 ; **p≤0.01 ; ***p≤0.001.

RB-1B inoculation induced a weak increase in nitrate production in very susceptible B13/B13 chickens one week after (TABLE 4). This increase remained nevertheless non-significant and disappeared three weeks post inoculation. The nitrate level after intravenous injection of LPS was used to evaluate the systemic response indicating the macrophage responding capacity to activation. LPS was able to increase strongly by 12-fold nitrate production in the serum of non-infected chickens 6 hours following intravenous injection. This inducing capacity of LPS on serum nitrate production remained unchanged or slighly reduced one week after RB-1B challenge in untreated chickens and in chickens pretreated with fowlpox virus. By contrast RB-1B-inoculated chickens exhibited exacerbated LPS-stimulated nitrate response in the serum three weeks following challenge. But none of the fowlpox pretreatments was able to modify such a response.

Example IV

36 Two-week-old B13/B13 chickens were divided in three groups of 12 : one group was inoculated with Fp/cMGF, one group with Fp/M3 vector (10^5 PFU per chicken) and the third group was not treated. They were challenged with RB-1B 7 days after fp

inoculation. RB-1B viremia was determined by semi-quantitative PCR 3 weeks after challenge. Mortality and macroscopic tumor incidence was also scored. A second group of 36 chickens were treated identically, except that vaccination with HVT was performed 3 days before RB-1B challenge. HVT vaccine was purchased from Fort-Dodge Santé Animale (France). 10^3 PFU were inoculated intramuscularly per chicken. All these chickens were sacrified 13 weeks after RB-1B challenge.

All non-vaccinated chickens died by 5 weeks after RB-1B challenge. All remaining HVT-vaccinated chickens were sacrificed 13 weeks after RB-1B challenge. Macroscopic tumors were scored and are located mainly in the liver, spleen, kidneys and others sites (heart, gonads, inoculation site)

TABLE 5.

Effect of fp/M3 and fp/cMGF on tumor incidence in RB-1B inoculated chickens

	Tumor incidence % (tumor score)				
	Total (%)	Liver	Spleen	Kidney	Others
5 weeks p.i					
RB-1B	92%	42% (++)	50% (+++)	68% (++++)	17% (+)
RB-1B + fp/M3	84%	34% (++)	20% (+)	73% (++++)	27% (++)
RB-1B + fp/cMGF	60%	9% (+)	9% (++)	60% (+++)	27% (+)
13 weeks p.i					
RB-1B +HVT	**50%**	11% (+)	11% (+)	**55% (++)**	0%
RB-1B +fp/M3 +HVT	**58%**	0%	44% (+)	**60% (++)**	11% (+)
RB-1B + fp/cMGF+HVT	**8%**	0%	0%	**8% (+)**	0%

Semi-quantitative PCR from total blood for determination of MDV viremia

For the extraction of DNA from blood, 50 µl of blood was mixed with 50 µl of 3% Sodium citrate, and then mixed with 500 µl lysis buffer (1% Saponin, Sigma). DNA was extracted from pelleted nuclei with 500 µl TE buffer (10 mM Tris, 1 mM EDTA) and 10 µl Proteinase K (20 mg/ml). The DNA was precipitated with cold ethanol in the

presence of 2 M Sodium acetate and centrifuged for 15 min at 10000 x g. The pellet was washed twice with 70% ethanol and dried under vacuum. The DNA was then resuspended in 250 µl TE buffer and stored at 4°C.

The semi-quantitative PCR assay was performed according to Bumstead et al (1997) with slight modifications. The primers used were chosen from sequence data for the ICP4 region of the MDV genome. The primers amplify a product of 329 bp from RB-1B.

Primer 1 : HEX-GATCGCCCACCACGATTACTACCT SEQ ID NO:14

Primer 2 : AATGAGCGAACTGCCTCACACAAC SEQ ID NO:15

Control primers were derived from the sequence of chicken Fas (Genbank Accession no. J04485) selected to give a product of 147 bp.

Primer 3 : TET-CTGATACAAGCAGGCAGAGC SEQ ID NO:16

Primer 4 : TGGTTGGATGGAGCAACTGG SEQ ID NO:17

Fluorescent primers (1,3) and non-fluorescent primers (2,4) were synthesized and HPLC modified by Eurogentec (France). The PCR reactions were carried out using 5 µl of total blood preparation, 10 pmol of each primer and 1.25 U Thermus aquaticus DNA polymerase (Promega, France) in a total volume of 25 µl. Amplification was carried out over 30 cycles, each consisting of 1 min at 94°C, 1 min at 62°C and 1 min at 72°C. After the final cycle, the elongation phase at 72°C was extended to 10 min. Two microliters of the PCR products were dried and resuspended in 0.5 µl Tamra 500 (Size standards available for fluorescent analysis, Amresco, France) and 20 µl formamide (Amresco, France). For quantification analysis of both fluorescence in each tube, the fluorescent peak heights were determined using ABI PRISM Genescan software. A positive RB-1B control extracted from feather follicules was always introduced as comparison and repeatedly gave the same results.

Viremia was tested directly on total blood using of a semi-quantitative PCR technique. The results (Shown in Fig 3) displayed more heterogeneity than for isolated blood leucocyte co-culture on fibroblasts. Nevertheless, the results led to a similar conclusion.

Control chickens infected with MDV and chickens inoculated with fp/M3 vector and then with RB-1B exhibited the same general pattern of viremia distribution. By contrast fp/cMGF-treated chickens showed a striking reduction in viremia.

All B13/B13 chickens inoculated with RB-1B died by five weeks following (FIG. 4). Fp/M3 inoculation seven days before MDV challenge slightly modified the timing of mortality, 50% mortality being reached one week later than for control chickens, however, all chickens were also dead by 5 weeks post challenge. fp/cMGF pretreatment delayed mortality even further, with 50% mortality being reached two weeks later than control chickens. Nevertheless, all fp/cMGF chickens died by 7 weeks after RB-1B challenge. Vaccination with HVT three days before RB-1B challenge reduced the mortality rate to 33% at 13 weeks, and this was not altered by co-treatment with fp/M13. However, co-treatment with fp/cMGF and HVT prevented mortality altogether. Tumor incidence was also affected by fp/cMGF treatment and this closely reflected the effects seen in mortality results. Ninety-two percent of RB-1B-infected control chickens had macroscopic tumors the spleen, liver and kidneys being the major site of tumor localisation (TABLE 5). The overall picture of tumor distribution was not greatly modified following pretreatment with fp/M3, except for reduction in spleen tumor burden at 5 weeks. In contrast, fp/cMGF pretreatment resulted in a dramatic 6-fold reduction the total tumor incidence at 13 weeks, eliminating all but the kidney tumours.

EXAMPLE V

Injection of cytokine polypeptide *in ovo*

This procedure describes the technique to be used for manual egg injection of test materials. Alternatively, eggs can be injected using an automated Inovoject® system.

a)Egg candling

Eggs are maintained under warm room conditions during candling and transfer, if possible. It is best not to keep eggs out of the incubator/hatcher for more than three hours. Eggs are candled by illumination of egg contents with an egg candler in a darkened room. Eggs that have a clear demarcation between air cell and embryo, a rosy

color, obvious veins or that show movement, are retained. Eggs that are clear (infertile), nonviable, cracked, excessively dirty, have green discoloration, or are upside down (no air cell in blunt end of egg), are discarded.

b) Needle/punch and egg sanitizer preparation

An 18 gauge 1.5 inch needle is inserted through a rubber stopper so that the needle cannot penetrate to a depth beyond 2mm. A 0.5% chlorine solution is prepared by 1:10 (v/v) dilution of 5.25% sodium hypochlorite (commercial bleach).

c) Administration of cytokine polypeptide *in ovo*

The blunt end of the egg is swabbed with a 0.5% chlorine solution (1:10 dilution of commercial bleach [5.25% sodium hypochlorite]). All eggs are manually punched with an 18 gauge needle attached to a rubber stopper. The cytokine preparation is delivered 2.5 cm through the blunt end of the egg, below the air cell membrane, using a 20 gauge x 1" needle attached to a 1 ml tuberculin syringe. The injection needle is sterilized between deliveries to each egg by punching into 1.5" thick sponge soaked in 0.5% chlorine solution. New needles and syringes are used each time the sample is withdrawn from the original container. Dose volume for *in ovo* administration is usually 100 ul (with 1 ml syringe). The injection hole is then covered using nail varnish and the injected eggs incubated in a hatcher. Administration by *in ovo* injection as described herein does not reduce hatch ability of eggs.

EXAMPLE VI

Expression of recombinant ChIFNγ in CK cells.

a) Construction of a recombinant FAV vector expressing avian IFNγ

(i) FAV vector sequences:

The 17.1 kb nucleotide sequence of the right-hand end of FAV serotype 8 (FAV8) is set forth in SEQ ID NO: 11. The right-hand end was identified by cloning and sequencing three restriction fragments of FAV8 (CFA40) as follows:

1. **Plasmid pJJ383**, deposited under the provisions of The Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (hereinafter "The Budapest Treaty") with Australian Government Analytical Laboratories at 1 Suakin Street, Pymble, New South Wales 2073, Australia on 11 November, 1999, and assigned AGAL Accession No. NM99/08170. Plasmid pJJ383 contains a *Nhe*I fragment of 8.5 kb in length derived from the FAV8 right-hand end, cloned into the vector pGEM-11f(+/-);

2. **Plasmid pJJ698**, deposited under the provisions of The Budapest Treaty with Australian Government Analytical Laboratories at 1 Suakin Street, Pymble, New South Wales 2073, Australia on 11 November, 1999, and assigned AGAL Accession No. NM99/08172. Plasmid pJJ698 contains a *Bgl*II fragment of 7.5 kb in length derived from the FAV8 right-hand end, cloned into the vector pUC18; and

3. **Plasmid pJJ407**, deposited under the provisions of The Budapest Treaty with Australian Government Analytical Laboratories at 1 Suakin Street, Pymble, New South Wales 2073, Australia on 11 November, 1999, and assigned AGAL Accession No. NM99/08173. Plasmid pJJ407 contains a *Bgl*II fragment of 1.7 kb in length derived from the FAV8 right-hand end, cloned into the vector pUC18.

(ii) Avian IFNγ expression cassette

An expression cassette was produced to facilitate the expression of IFNγ from recombinant FAV, said cassette comprising the FAV MLP sequence (Johnson, D.C., *et al.* (1988) *Virology 164,* 1-14) operably connected to the ChIFNγ coding region, and placed upstream of the SV40 transcription termination signal, by cloning the cassette into the *Not*I site of the bacterial plasmid vector pUC18. The plasmid produced that contains this expression cassette was designated plasmid pJJ427.

Plasmid pJJ427 was deposited under the provisions of The Budapest Treaty with Australian Government Analytical Laboratories at 1 Suakin Street, Pymble, New South

Wales 2073, Australia on 11 November, 1999, and assigned AGAL Accession No. NM99/08169.

The *Not*I site in plasmid pJJ427 is also useful for subsequent insertion of the expression cassette into FAV8 sequences, either directly, or following end-filling and blunt-end ligation.

(iii) Recombinant FAV containing the avian IFNγ expression cassette

There are unique *Xba*I, *Sna*BI and *Sma*I sites in the *Nhe*I fragment of plasmid pJJ383 to facilitate the insertion of foreign DNA.

In one vector construct, the 1.3 kb region between the unique *Sna*BI and *Sma*I sites of plasmid pJJ383 was deleted and replaced by the IFNγ expression cassette of plasmid pJJ427. The resultant plasmid, designated pJJ464, was deposited under the provisions of The Budapest Treaty with Australian Government Analytical Laboratories at 1 Suakin Street, Pymble, New South Wales 2073, Australia on 11 November, 1999, and assigned AGAL Accession No. NM99/08171.

In a second vector construct, the 2.2 kb *Xba*I-*Sna*BI fragment of pJJ383 was deleted and replaced by the expression cassette of plasmid pJJ427. The resultant plasmid, designated pJJ677, was deposited under the provisions of The Budapest Treaty with Australian Government Analytical Laboratories at 1 Suakin Street, Pymble, New South Wales 2073, Australia on 11 November, 1999, and assigned AGAL Accession No. NM99/08174.

In a third vector construct, the 50 bp region between two *Spe*I sites of pJJ383 was deleted and replaced by the expression cassette of plasmid pJJ427, leaving most of the FAV8 vector sequence intact. The resultant plasmid, designated pJJ486, was deposited under the provisions of The Budapest Treaty with Australian Government Analytical Laboratories at 1 Suakin Street, Pymble, New South Wales 2073, Australia on 11 November, 1999, and assigned AGAL Accession No. NM99/08175.

The deposits referred to herein will be maintained under the terms of The Budapest Treaty and are provided merely as a convenience to those skilled in the art and not as an admission that any one or more of said deposits is required under 35 U.S.C. §112.

To produce recombinant FAV expressing ChIFNγ , each of the three plasmids pJJ464, pJJ677, and pJJ486, containing the expressible ChIFNγ encoding region, was transfected with *Spe*I-digested FAV viral genomic DNA. The recombinant FAVs were plaque-purified and characterized by Southern blotting and PCR using standard procedures.

b) Transcriptional mapping

To confirm expression of IFNγ from recombinant FAV, mRNA was isolated from infected cell cultures at 6 hr and 20 hr post-infection with either wild-type FAV8 or recombinant FAV8 comprising the IFNγ coding region produced as described *supra*. The mRNA was purified using Qiagen Direct mRNA Maxi kit, and transferred directly to nylon membranes using the Ambion Northern Max-Gly kit. Fragments derived from the right-hand end of the FAV8 genome were probed, using the protein-coding region of the ChIFNγ cDNA, radiolabelled with ^{32}P, as a probe. This analysis confirmed the presence of ChIFNγ transcripts in the recombinant FAV only.

To confirm these results, mRNA was also used as a substrate for RT-PCR employing the Promega Reverse Transcription System, employing primers that were specific to ChIFNγ . This analysis confirmed the presence of ChIFNγ transcripts in recombinant FAV-ChIFNγ-infected cells at 20 hr post-infection. The amplified product was also confirmed as ChIFNγ , by subsequent sequence determination.

c) Biological activity of recombinant ChIFNγ produced in recombinant FAV-ChIFNγ-infected cells

The biological activity of rChIFNγ was determined using the nitrite release assay. Two-fold serial dilutions of test supernatants from cultures of CK cells that were infected with either wild-type FAV8 or with r FAV-ChIFNγ were made in duplicate wells of 96 well plates. HD11 cells were added to each well and the plates incubated at 37 C. After 24 hr, 50 µl of culture supernatant was added to 100 µl of Griess Reagent and the absorbance determined at 540 nm. Duplicate cultures were also incubated in the presence or absence of 1% (v/V) rabbit anti-ChIFNγ serum, which blocks ChIFNγ , but not Type I molecules. The results indicate that rChIFNγ is expressed and is biologically-active.

EXAMPLE VII

Enhancement of immune reactions using recombinant avian IFNγ polypeptides

1. ChIFNγ as an adjuvant

Four Groups (n = 10) of 3-week old SPF chickens were injected intramuscularly (i/m) with either 0.2 or 0.02 ml of sheep red blood cells (SRBC). One group at each dose was also injected intra-peritoneally with 500 Units of recombinant ChIFNγ the day before and on the day of immunization. Birds were bled weekly and haemaglutination titres of the sera were determined.

Chickens were injected with SRBC (with or without recombinant ChIFNγ) and weekly haemaglutination (HA) titres of the sera were determined. Treatment with recombinant ChIFN□ resulted in a higher mean HA titre, a prolonged antibody response and increased the effectiveness of the low dose of antigen. This indicates that recombinant ChIFNγ is an effective adjuvant.

2. Effect of recombinant ChIFNγ on infection with IBDV *in vivo*

One group (n = 10) of three-week old SPF chickens was injected intraperitoneally with 500 Units of recombinant ChIFNγ on 2 consecutive days and another group of control birds (n =10) was injected with diluent alone. Both groups of birds were infected intra-ocularly with Infectious Bursal Disease Virus (IBDV). Birds were sacrificed 7 days later and the bursa and whole body weights were determined.

Birds injected with recombinant ChIFNγ displayed an enhanced ratio of body: bursa weight from a mean of 1.36 to 1.51 indicating that recombinant ChIFNγ was effective in reducing virus growth *in vivo*.

The effect of recombinant ChIFNγ to protect CEFs from infection with IBDV *in vitro* was also measured. CEFs were prepared as described for the CEF interferon assay and recombinant ChIFNγ and IBDV were added to the cultures together. Cell survival was measured 3 days later on a scale of 0 to 4, where 0 represents the level of cell survival observed in the presence IBDV and the absence of IFNγ(< 5% cell survival) and 4 represents the level of cell survival observed in the absence of IBDV (> 90 % cell survival).Recombinant ChIFNγ was effective in protecting CEFs from infection with IBDV *in vitro*.

In conclusion, recombinant ChIFNγ has been shown to effective in the prevention of infection by IBDV both *in vivo* and *in vitro*.

b) Use of recombinant FAV to deliver rChIFNγ

Commercial broiler chickens at 1 day, 3 days, 6 days, or 10 days post-hatching, were infected by eye-drop with rFAV-ChIFNγ produced from plasmid pJJ464, as described *supra*. Birds were housed in positive pressure isolators and maintained on a constant feed regime. Birds were weighed weekly for a period of 7 weeks. All treated birds receiving rFAV-ChIFNγ showed increased weight gains which were significantly different from control untreated birds in paired t-tests carried out at 34 days and 42 days

post-treatment . The most significant weight gains were observed in birds infected at 3 days or later post-hatching with recombinant FAV expressing ChIFNγ , compared to uninfected birds. Less-significant differences were observed between birds that had been infected at different ages post-hatching. Similar effects are observed using rFAV derived from plasmid pJJ486.

c) Use of recombinant FAV to deliver rChIFNγ

Commercial broiler chickens at 1 day or 6 days post-hatching were infected by eye-drop with rFAV-ChIFNγ produced from plasmid pJJ464, as described *supra.* At 7 days post-hatch, birds were challenged with 5,000 sporulated oocysts of *E. acervulina.* A control group remained unchallenged. Birds were housed in positive pressure isolators and maintained on a constant feed regime. Birds were weighed daily post-challenge. All treated birds receiving rFAV-ChIFNγ showed increased weight gains compared to controls that were not treated with ChIFNγ, and those birds maintained these weight gains under coccidiosis challenge, compared to birds that were not treated with ChIFNγ . Similar effects are observed using rFAV derived from plasmid pJJ486.

EXAMPLE VIII

Construction of plasmid Vaccine containing MDV gB gene, MDV gD gene, IBDV VP2 gene, IBV S1 gene, IBV M gene, or IBV N gene

After culturing, the supernatant and the lysed cells from virally infected culture are harvested and the entire viral suspension is centrifuged at 1000 g for 10 minutes at +4.degree. C. so as to remove the cellular debris. The viral particles are then harvested by ultracentrifugation at 400,000 g for 1 hour at +4.degree. C. The pellet is taken up in a minimum volume of buffer (10 mM Tris, 1 mM EDTA). This concentrated viral suspension is treated with proteinase K (100 .mu.g/ml final) in the presence of sodium dodecyl sulphate (SDS) (0.5% final) for 2 hours at 37.degree. C. The viral DNA is then extracted with a phenol/chloroform mixture and then precipitated with 2 volumes of absolute ethanol. After leaving overnight at -20.degree. C., the DNA is centrifuged at 10,000 g for 15 minutes at +4.degree. C. The DNA pellet is dried and then taken up in a

minimum volume of sterile ultrapure water. It can then be digested with restriction enzymes.

The RNA viruses were purified according to techniques well known to persons skilled in the art. The genomic viral RNA of each virus was then isolated using the "guanidium thiocyanate/phenol-chloroform" extraction technique described by P. Chromczynski and N. Sacchi (Anal. Biochem., 1987. 162, 156-159).

All the constructions of plasmids were carried out using the standard molecular biology techniques described by J. Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). All the restriction fragments were isolated using the "Geneclean" kit (BIO 101 Inc. La Jolla, Calif.).

Specific oligonucleotides (comprising restriction sites at their 5' ends to facilitate the cloning of the amplified fragments) were synthesized such that they completely cover the coding regions of the genes which are to be amplified . The reverse transcription (RT) reaction and the polymerase chain reaction (PCR) were carried out according to standard techniques (Sambrook J. et al., 1989). Each RT-PCR reaction was performed with a pair of specific amplimers and taking, as template, the viral genomic RNA extracted. The complementary DNA amplified was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) before being digested with restriction enzymes.

The plasmid pVR1012 was obtained from Vical Inc., San Diego, Calif., USA. Its construction has been described in J. Hartikka et al. (Human Gene Therapy, 1996, 7, 1205-1217).

For the preparation of the plasmids, any technique may be used which makes it possible to obtain a suspension of purified plasmids predominantly in the supercoiled form. These techniques are well known to persons skilled in the art. There may be mentioned

in particular the alkaline lysis technique followed by two successive ultracentrifugations on a caesium chloride gradient in the presence of ethidium bromide as described in J. Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). Reference may also be made to patent applications PCT WO 95/21250 and PCT WO 96/02658 which describe methods for producing, on an industrial scale, plasmids which can be used for vaccination. For the purposes of the manufacture of vaccines the purified plasmids are resuspended so as to obtain solutions at a high concentration (>2 mg/ml) which are compatible with storage. To do this the plasmids are resuspended either in ultrapure water or in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

The various plasmids necessary for the manufacture of an associated vaccine are mixed starting with their concentrated solutions. The mixtures are prepared such that the final concentration of each plasmid corresponds to the effective dose of each plasmid. The solutions which can be used to adjust the final concentration of the vaccine may be either a 0.9% NaCl solution, or PBS buffer.

EXAMPLE IX

Production and purification of recombinant cMGF protein

To construct recombinant plasmid pET22b to express a 6xHis-tagged form of cMGF, cMGF cDNA was isolated as PCR product by using primers 1 and 2. Primer sequences were: primer 1, 5' TAATACGACTCACTAT; primer 2, 5'AGGAAGCTGAGTTGGCTGCTG. Several clones were isolated and the nucleotide sequence was determined by DNA sequencing. Plasmid DNA containing the correct sequence was used for transformation of the T7 polymerase expression BL21 (DE3) competent cells. A bacterial clone with high expression of the recombinant cMGF protein was used for large scale protein preparation.

Induction of cMGF synthesis by IPTG and purification of recombinant protein on a His-Bind Kit was performed according to the manufacturer's procedure (Qiagen, Courtaboeuf,

France) with some modifications. Briefly, cells were grown in 1 liter B medium to a density of A600 = 0.6 and expression of recombinant protein was induced by addition of 1 mM IPTG. Two hours later, they were harvested and lysed in 25 ml lysis buffer (6M urea, 20 mM tris-HCl pH 8, 500mM NaCl, 3 mM imidazole, 0.05% triton) at 4°C (24 hours) with constant stirring. Insoluble material was removed by centrifugation (10 000g, 20 min) and the supernatant was passed over a nickel affinity column specifically to purify multiple histidine-containing proteins. The sample was eluted using an imidazole step gradient (100-500nM), dialyzed against phosphate-buffered saline (PBS), concentrated using an Ultrafree concentrator (Millipore, Bedford, France), and tested for biological activity *in vivo*. The quality of protein purification was checked on acrylamide gel. Prepared protein samples were also tested to be free of putative contaminating LPS using the Limulus assay (Sigma-Aldrich, St Quentin Fallavier, France) and pooled before use. Protein concentration of the recombinant protein preparation was estimated using Bradford assay which was then sterile filtrated and stored in aliquots at - 80C.

EXAMPLE X

Injection of recombinant CMGF protein

A. Effect of treatment with rcMGF inoculation on number and function of monocytes

Three histocompatible B13/B13 chickens were inoculated intramuscularly with one or two or three daily injections of 50µg rcMGF respectively, at 10 days of age, and one chicken was left untreated as control. The increase in blood monocytes and nitric oxide (NO_2^- + NO_3^-) levels in serum were measured 3 days after the last injection.

The activity of rcMGF protein produced in *E. coli* and purified using His-tag addition was tested *in vivo* in B^{13}/B^{13} chickens. Treatment with 50µg (per chicken) injection of cMGF given one, two or three times at one day intervals induced an increase in number of aggregated monocytes in blood compared to control non-treated chicken by 3 days after the last injection, identified by the specific monoclonal antibody 68.1. The effects of cMGF treatment were also tested on NO production in the same chickens. Serum nitrate reflects systemic NO production . Inoculation of 150 µg rcMGF was able to induce a

significant (2-fold) increase in serum nitrate background in the blood 3 days after the last inoculation compared to non-treated control. The increase in background nitrate levels was also observed with 100 µg rcMGF in experiment B. Nitrate levels after intravenous injection of LPS were used to evaluate the systemic response indicating macrophage responding capacity to activation (Smith SR, Manfra D, Davies L, Terminelli C, Denhardt G, Donkin J. Elevated levels of NO in both unchallenged and LPS-challenged C. parvum-primed mice are attributable to the activity of a cytokine-inducible isoform of iNOS. J Leukoc Biol 1997;61:24-32.) LPS was able to increase nitrate production in the serum of non-treated control chickens 6 hours after intravenous injection (4.5-fold). This enhanced response to LPS was strongly exacerbated following inoculation from 50 to 150 µg rcMGF per chicken, resulting in a significant (about 2-fold) increase in serum nitrate production compared to control chickens. Treatment with rcMGF was thus effective in enhancing NO production as early as 3 days later.

B. Effects of treatment with rcMGF and vaccination in MDV-infected chickens.

Nitrate response

50 B13/B13 chickens were inoculated with 2 daily injections of 50µg rcMGF and 50 chickens were left untreated. Half of each group were vaccinated with HVT on the second day of treatment with rcMGF. Three days later, all chickens (except 5 from each group to be tested just before the RB-1B challenge) were then challenged with RB-1B to measure RB-1B viremia in blood leukocytes in the fibroblast assay and nitric oxide (NO_2^- + NO_3^-) levels in serum on day 7 and day 21 post infection (5 chickens per group and per day for each test). Ten chickens were kept as uninfected controls.

RB-1B inoculation induced a significant increase in nitrate production in B13/B13 chickens one week later. This increase persisted for three weeks post inoculation.

Nitrate levels after intravenous injection of LPS were also used to evaluate the systemic response indicating that macrophages respond to activation after MDV infection. LPS was able to increase nitrate production considerably in the serum of non-infected chickens 6

hours following intravenous injection (about 7 times above background levels). LPS-stimulated serum nitrate levels in RB-1B inoculated chickens were twice as high as those of non-infected control chickens one to three weeks following challenge. Treatment with rcMGF before MDV infection did not prevent the increase in spontaneous and LPS-induced blood nitrate levels.

Vaccination with HVT reduced the increase in nitrate levels after RB-1B challenge and the response to LPS from one to three weeks post infection. Additional concomitant treatment with cMGF was able to reduce the blood nitrate levels further after RB-1B challenge, and at the same time was able to increase slightly the capacity to respond to LPS.

In vivo MDV replication

RB-1B viremia was evaluated one and three weeks following inoculation of RB-1B by co-culture of blood leukocytes on embryo fibroblasts. MDV is characterized by strong T-cell association and is not found in the supernatant or serum (Calnek BW. Marek's disease--a model for herpesvirus oncology. Crit Rev Microbiol 1986;12:293-320.). The number of PFU was measured one week after contact of blood leukocytes with fibroblasts. Treatment with rcMGF resulted in a significant reduction (3 times less) in the RB-1B PFU recovered from blood leukocytes one week after challenge. By 3 weeks post challenge, the recovery of RB-1B PFU from blood leukocytes of rcMGF-treated chickens increased 2-fold, but remained still significantly lower than for RB-1B-infected non-treated chickens. In chickens vaccinated with HVT, a herpes virus isolated from turkeys and not pathogenic for chickens, HVT was recovered from blood leukocytes 1 and 3 weeks following inoculation. Nevertheless HVT PFU could not be distinguished easily from RB-1B PFU, especially in a mixture of the two viruses. Consequently, the number of PFU counted included both viruses when chickens were inoculated with both. However vaccination with HVT drastically reduced the total number of PFU recovered from blood leukocytes (44%) one week post challenge with RB-1B compared with non-vaccinated and RB-1B-challenged chickens after inoculation of RB-1B. The decrease was even more pronounced (73%) after three weeks. Treatment with rcMGF in addition to vaccination did not further decrease the

number of PFU recovered from blood leukocytes at the times tested post challenge with RB-1B.

C. Effects of treatment with rcMGF and vaccination on tumor incidence and mortality following MDV infection

100 B13/B13 chickens were divided into four groups : one group was left untreated, the second group was treated with 2x50 μg cMGF, the third was vaccinated with HVT and the fourth group was treated with rcMGF and vaccinated with HVT. All chickens were then challenged with RB-1B 3 days after vaccination with HVT. Mortality and the incidence of macroscopic tumors were scored. A qualitative tumor score was ascribed per organ : + for one or few small tumors to ++++ for large scale tumor infiltration. All the vaccinated chickens were killed 10 weeks following RB-1B challenge.

All unvaccinated/untreated B13/B13 chickens inoculated with RB-1B at 15 days of age had died by six weeks following infection (Fig. 5). Treatment with rcMGF 3 days before MDV challenge slightly modified the timing of mortality, 50% mortality being reached one week later than for control chickens.

Eighty-one percent of unvaccinated/untreated RB-1B-infected control chickens had macroscopic tumors, the spleen, liver and kidneys being the major sites of tumor localization. By contrast tumor incidence and tumor burden, especially in the spleen and kidneys, were greatly reduced in rcMGF-treated chickens (Table 6).

Vaccination with HVT 3 days before RB-1B challenge also reduced mortality significantly compared to non-vaccinated chickens. By 10 weeks post challenge, 60% of vaccinated chickens were still alive. Tumor incidence was also affected by vaccination, with reduction in the number of chickens with macroscopic visceral tumors (by about half) and overall reduction of tumor development in the spleen, liver and kidneys (by 50 to 70%) (Table 6). Even further reduction in numbers of chickens with macroscopic visceral tumors and splenic tumors was observed in cMGF treated, vaccinated chickens compared to chickens

which were only vaccinated. Thus administration of rcMGF at the time of vaccination had enhanced beneficial effects in reducing tumor development.

Table 6:
Effects of HVT vaccination and rcMGF treatment on visceral tumor development in highly susceptible B^{13}/B^{13} chickens after inoculation of RB-1B 3 days later

	% tumor incidence (score)				
	Total	**Liver**	**Spleen**	**Kidneys**	**Others**
RB-1B	81%	19% (+++)	48 % (+++)	67% (+++)	0
rcMGF+RB-1B	48 %	22 % (++)	13 % (+++)	48% (+++)	4%
HVT+RB-1B	45 %	5 % (+)	22 % (+)	32% (++)	14%
rcMGF+HVT+RB-1B	20 %	4% (+)	8% (+)	20% (++)	8%

Qualitative tumor score was ascribed per organ : + for one or few small tumors to +++ for numerous and/or large tumors.

D. Effects of treatment with rcMGF and vaccination on tumor incidence and mortality following MDV infection

One group of 30 chickens was challenged with RB-1B at 3 days old. Two other groups of 30 were vaccinated with HVT at hatching, treated with 50 µg of rcMGF or left untreated, and then challenged 3 days later with RB-1B. Mortality and tumor development were recorded.

For very susceptible B^{13}/B^{13} chickens, vaccination with HVT at 3 days of age did not protect effectively against high dose challenge with very virulent RB-1B. All challenged control chickens were dead by 26 days p.i., (Fig 6). The majority of these chickens developed large visceral tumors, with 60% or more with severe tumor development in the

liver, spleen and kidneys and about 40% in the gonads (Table 7). 12% of vaccinated chickens survived and tumor development was also severe, although in a slightly less extent compared to infected control chickens particularly in the kidneys and gonads. Treatment with rcMGF had a striking effect in vaccinated chickens, significantly delaying mortality ($p \leq 0.05$ at days 18 and 20 post challenge), compared to chickens which were only vaccinated. In addition, tumor burden was reduced in all major localizations, especially in the kidneys and gonads.

Table 7

Effects of HVT vaccination and rcMGF treatment at day 3 on visceral tumor development in very susceptible B^{13}/B^{13} chickens inoculated with RB-1B.

	% tumor incidence (score)					
	Total	**Liver**	**Spleen**	**Kidneys**	**Gonads**	**Others**
RB-1B	91.2	58.8 (++++ 23.5 +++ 17.6)	79.4 (++++ 29.4 +++ 35.3)	70.6 (++++ 5.9 +++ 32.3)	44.1 (++ 14.7)	8.8
HVT+RB-1B	90.9	69.7 (++++ 12.1 +++ 24.2)	66.7 (++++ 12.2 +++ 39.4)	54.5 (+++ 27.3)	24.2 (++ 12.12)	18.2
HVT+rcMGF+ RB-1B	92.9	50.0 (++++ 7.1 +++ 17.9)	64.3 (++++ 14.3 +++ 17.9)	39.3 (+++ 7.1)	14.3	14.3

Qualitative tumor score was ascribed per organ : + for one or few small tumors to ++++ for large scale tumor infiltration.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of treatment or prophylaxis of avian pathogenic disease in a bird, said method comprising administering thereto an effective amount of one or more avian cytokine polypeptides for a time and under conditions sufficient to stimulate the immune response of said bird to an antigen.

2. A method according to claim 1 wherein the cytokine polypeptide is a chicken cytokine.

3. A method according to claim 1 wherein the cytokine polypeptide is selected from a colony stimulating factor, interferon and interleukin.

4. A method according to claim 1 wherein the cytokine polypeptide is selected from the group consisting of myelomonocyte growth factor, interferon γ, IL-2 , IL-15, IL-18 and subunits thereof.

5. A method according to claim 1 wherein the cytokine polypeptide is selected from the group consisting of cMGF, chIFNγ, chIL-2, chIL-15 and chIL-18 or a derivative thereof.

6. A method according to claim 1 wherein the cytokine polypeptide is selected from the group consisting of:

   (a) a polypeptide having the amino acid sequence set forth in any one of SEQ ID NOs:2, 4, 6, 8 or 10;

   (b) a polypeptide having the amino acid sequence set forth as the mature protein region of any one of SEQ ID NOs: 2, 4, 6, 8 or10;

   (c) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence, wherein said nucleic acid molecule hybridizes under conditions of at least moderate stringency with a probe having a sequence complementary to at least 50 contiguous nucleotides of SEQ ID NOs:1, 3, 5, 7 or 9;

(d) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence that is degenerate with a DNA molecule according to (c);

(g) a polypeptide comprising at least 10 contiguous amino acids of any one of SEQ ID NOs: 2, 4, 6, 8 or 10, wherein said polypeptide has immunomodulatory activity; and

(h) a homologue, analogue or derivative of any of the polypeptides of (a) to (e) wherein said polypeptide has immunomodulatory activity.

7. A method according to claim 1 wherein the cytokine polypeptide is selected from the group consisting of a polypeptide comprising the amino acid sequences set forth in any one of SEQ ID NOs: 2, 4, 6, 8 and 10.

8. A method according to claim 6 wherein the nucleotide sequence is substantially the same as or complementary to the nucleotide sequence set forth in SEQ ID NOs:1, 3, 5, 7 or 9 or a homologue, analogue or derivative thereof.

9. A method according to claim 6 wherein the nucleic acid molecule is operably connected to a promoter derived from an endogenous cytokine polypeptide gene or another, which regulates the expression of the cytokine polypeptide sequence contained therein and is capable of conferring expression in the cell, tissue, organ or organism in which expression is desired.

10. A method according to claim 9 wherein the promoter is a highly-active constitutive promoter selected from the group consisting of SV40 major later promoter, FAV major later promoter, cytomegalovirus immediate early promoter, or human adenovirus major late promoter.

11. A method according to claim 1 wherein the cytokine polypeptide is administered directly.

12. A method according to claim 1 wherein the cytokine polypeptide is administered by the delivery of a nucleic acid molecule encoding said cytokine polypeptide.

13. A method according to claim 12 wherein the delivery is by direct DNA injection, attenuated virus, recombinant viral vector, mycoplasma vector or bacterial vector.

14. A method according to claim 12 wherein the delivery is by viral .

15. A method according to claim 12 wherein the delivery by a fowlpox vector (FPV) a fowl adenovirus vector (FAV), newcastle disease virus (NDV) vector or marek's disease virus (MDV) vector.

16. A method according to claim 1 wherein the route of administration is selected from the group consisting of intra-peritoneal, intra-ocular, intravenous, intramuscular, intra nasal, oral, sub-cutaneous, transcutaneous and intradermal.

17. A method according to claim 1 wherein the mode of administration is by injection, aerosol or oral ingestion.

18. A method according to claim 1 wherein the mode of administration is neubilization, spray or drinking water.

19. A method according to claim 1 wherein the cytokine polypeptide is administered during the first few days post hatching.

20. A method according to claim 1 wherein the cytokine polypeptide is administered after the first few days post hatching.

21. A method according to claim 1 wherein the cytokine polypeptide is administered *in ovo*.

22. A method according to claim 1 wherein the cytokine polypeptide enhances a cell mediated immune response.

23. A method according to claim 1 wherein the cytokine polypeptide induces or increases a humoral immune response.

24. A method according to claim 1 wherein the cytokine polypeptide enhances innate immunity.

25. A method according to claim 1 wherein the cytokine polypeptide enhances cytokine production.

26. A method according to claim 1 wherein the immune response inhibits viral replication.

27. A method according to claim 1 wherein the immune response decreases disease mortality.

28. A method according to claim 1 wherein the immune response decreases tumor incidence tumour size and/or number of tumours in the liver, spleen, kidneys, heart, gonads, or inoculation site.

29. A method according to claim 1 wherein the cytokine polypeptide alleviates pathogen related reduced growth performance.

30. A method according to claim 1 further comprising administration of an immunomodulator.

31. A method according to claim 30 wherein the cytokine polypeptide enhances the specific or non-specific immune response to the immunomodulator.

32. A method according to claim 30 wherein the immunomodulator is selected from the group consisting of an antigen, peptide, polypeptide, protein, vaccine, attenuated pathogen, polynucleotide vaccine, a complete gene or fragments, a live whole vaccine, an inactivated whole vaccine, a recombinant vaccine, hapten, adjuvant, organism, subunit vaccine, a disease-promoting agent, an infectious agent, a tumour a cancer-inducing agent and a nucleotide molecule encoding any of the aforementioned.

33. A method according to claim 30 wherein the immunomodulator provides a second cytokine polypeptide, wherein said second cytokine polypeptide acts to maintain, stimulate, suppress or repress the same or different aspects of the immune system as the first cytokine polypeptide.

34. A method according to claim 30 wherein the immunomodulator is selected from the group consisting of infectious bursal disease virus (IBDV), avian infectious bronchitis virus (IBV), avian infectious laryngeotracheitis virus (ILTV), Newcastle disease virus (NDV), Marek's Disease virus (MDV), chicken anemia virus (CAV) avian influenza virus (AIV), avian leukosis virus and pneumovirosis virus.

35. A method according to claim 30 wherein the immunomodulator is one or more selected from the group consisting of the S, M and N antigens of infectious

bronchitis virus, gB and gD antigens of avian infectious laryngeotracheitis virus, HN and F antigens of Newcastle disease virus, gB, gC and gD antigens of Marek's disease virus, C and NS1 antigens of chicken anaemia virus, HA, N and NP antigens of avian influenza virus, VP2 antigen of infectious Bursal disease virus, env and gag/pol antigens of avian leukosis virus, F and G antigens of pneumovirosis virus.

36. A method according to claim 30 wherein the immunomodulator is a Marek's disease vaccine.

37. A method according to claim 36 wherein the Marek's disease vaccine is one or more selected from the group consisting of attenuated strains of serotype 1 (such as Rispens), strains of serotype 2 (such as SB1), and HVT.

38. A method according to claim 30 wherein the cytokine polypeptide is administered together or separately with the immunomodulator.

39. A method according to claim 30 wherein the cytokine polypeptide and immunomodulator are delivered as a fusion molecule.

40. A method according to claim 1 wherein administration of the cytokine polypeptide is followed after a period of time with administration of an effective dose of a conventional vaccine.

41. A method according to claim 1 wherein administration of the cytokine polypeptide is preceded by administration of an effective dose of a conventional vaccine.

42. A method according to claim 1 wherein administration of the cytokine polypeptide is concurrent with administration of an effective dose of a conventional vaccine.

43. A method according to claim 1 wherein the immune response is desired against an infectious disease transmitted by virus, bacteria or Mycoplasma.

44. A method according to claim 43 wherein the infectious disease is an avian viral infectious agent.

45. A method according to claim 43 wherein the infectious disease is selected from the group consisting of infectious bursal disease virus (IBDV), avian infectious bronchitis virus (IBV), avian infectious laryngeotracheitis virus (ILTV), Newcastle

disease virus (NDV), Marek's Disease virus (MDV), chicken anemia virus (CAV) avian influenza virus (AIV), avian leukosis virus and pneumovirosis virus.

46. A method according to claim 43 wherein the infectious disease is caused by *E.coli, Salmonella* ssp. or *Eimeria* ssp..

47. A method according to claim 1 wherein said cytokine polypeptide is administered to a bird selected from the group consisting of:

(i) healthy birds that are susceptible to infection by Marek's disease virus.;

(ii) asymptomatic birds infected with Marek's disease virus.; and

(v) birds suffering from Marek's disease.

48. A method according to claim 1 wherein said bird is a poultry, domestic or game bird

49. A method according to claim 1 wherein said bird is selected from the group consisting of chickens, turkeys, bantams, quails, guinea fowl, ducks, geese, ostriches, emus, pigeons, canaries, budgerigars, parrots and finches.

50. A gene construct comprising:

(iii) a first nucleotide sequence encoding a cytokine polypeptide; and

(iv) a second nucleotide sequence encoding an immunomodulator

51. A gene construct according to claim 50 wherein the immunomodulator is an antigen.

52. A gene construct according to claim 50 wherein the construct further comprises a delivery vehicle comprising genetic sequences which facilitate delivery or replication of said gene construct.

53. A gene construct according to claim 52 wherein the delivery vehicle is an attenuated virus, recombinant virus, mycoplasma vector or bacterial vector.

54. A gene construct according to claim 52 wherein the delivery vehicle is a non-replicating, non-infectious viral vector.

55. A gene construct according to claim 50 wherein the nucleotide sequences may be present in the same transcription unit or in two different units, in different plasmids or in one and the same plasmid, in the same or separate delivery vehicles.

56. A vaccine composition for the treatment or prophylaxis of a bird against a pathogenic disease comprising an effective amount of :

(i) one or more avian cytokine polypeptides or nucleic acid molecule encoding same; and

(ii) an immunomodulator or nucleic acid molecule encoding same.

57. A vaccine composition according to claim 56 wherein the cytokine polypeptide is a chicken cytokine.

58. A vaccine composition according to claim 56 wherein the cytokine polypeptide is selected from a colony stimulating factor, interferon and interleukin.

59. A vaccine composition according to claim 56 wherein the cytokine polypeptide is selected from the group consisting of myelomonocyte growth factor, interferon γ, IL-2 , IL-15, IL-18 and subunits thereof.

60. A vaccine composition according to claim 56 wherein the cytokine polypeptide is selected from the group consisting of cMGF, chIFNγ, chIL-2, chIL-15 and chIL-18 or a derivative thereof.

61. A vaccine composition according to claim 56 wherein the cytokine polypeptide is selected from the group consisting of:

(a) a polypeptide having the amino acid sequence set forth in any one of SEQ ID NOs:2, 4, 6, 8 or 10;

(b) a polypeptide having the amino acid sequence set forth as the mature protein region of any one of SEQ ID NOs: 2, 4, 6, 8 or10;

(c) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence, wherein said nucleic acid molecule hybridizes under conditions of at least moderate stringency with a probe having a sequence complementary to at least 50 contiguous nucleotides of SEQ ID NOs:1, 3, 5, 7 or 9;

(d) a polypeptide encoded by a nucleic acid molecule having a nucleotide sequence that is degenerate with a DNA molecule according to (c);

(e) a polypeptide comprising at least 10 contiguous amino acids of any one of SEQ ID NOs: 2, 4, 6, 8 or 10, wherein said polypeptide has immunomodulatory activity; and

(f) a homologue, analogue or derivative of any of the polypeptides of (a) to (e) wherein said polypeptide has immunomodulatory activity.

62. A vaccine composition according to claim 56 wherein the cytokine polypeptide is selected from the group consisting of a polypeptide comprising the amino acid sequences set forth in any one of SEQ ID NOs: 2, 4, 6, 8 and 10.

63. A vaccine composition according to claim 56 wherein the nucleotide sequence is substantially the same as or complementary to the nucleotide sequence set forth in SEQ ID NOs:1, 3, 5, 7 or 9 or a homologue, analogue or derivative thereof .

64. A vaccine composition according to claim 56 wherein the immunomodulator is selected from the group consisting of an antigen, peptide, polypeptide, protein, vaccine, attenuated pathogen, polynucleotide vaccine, a complete gene or fragments, a live whole vaccine, an inactivated whole vaccine, a recombinant vaccine, hapten, adjuvant, organism, subunit vaccine, a disease-promoting agent, an infectious agent, a tumour a cancer-inducing agent.

65. A vaccine composition according to claim 64 wherein the antigen is selected from the group consisting of infectious bursal disease virus (IBDV), avian infectious bronchitis virus (IBV), avian infectious laryngeotracheitis virus (ILTV), Newcastle disease virus (NDV), Marek's Disease virus (MDV), chicken anemia virus (CAV) avian influenza virus (AIV), avian leukosis virus and pneumovirosis virus.

66. A vaccine composition according to claim 64 wherein the antigen is one or more selected from the group consisting of the S, M and N antigens of infectious bronchitis virus, gB and gD antigens of avian infectious laryngeotracheitis virus, HN and F antigens of Newcastle disease virus, gB, gC and gD antigens of Marek's disease virus, C and NS1 antigens of chicken anaemia virus, HA, N and NP antigens of avian influenza virus, VP2 antigen of infectious Bursal disease virus, env and gag/pol antigens of avian leukosis virus, F and G antigens of pneumovirosis virus.

67. A vaccine composition according to claim 64 wherein the antigen is a Marek's disease vaccine.

68. A vaccine composition according to claim 67 wherein the Marek's disease vaccine is one or more selected from the group consisting of attenuated strains of serotype 1 (such as Rispens), strains of serotype 2 (such as SB1), and HVT.

69. A vaccine composition according to claim 56 wherein the nucleic acid molecules encoding the cytokine polypeptide and immunomodulator are present in the same transcription unit or in two different units, in different plasmids or in one and the same plasmid, in the same or separate delivery vehicles.

70. A vaccine composition according to claim 69 wherein the delivery vehicle is an attenuated virus, recombinant virus, mycoplasma vector or bacterial vector.

71. A vaccine composition according to claim 69 wherein the delivery vehicle is a non-replicating, non-infectious viral vector.

72. A vaccine composition according to claim 56 wherein the cytokine polypeptide and immunomodulator are a fusion molecule.

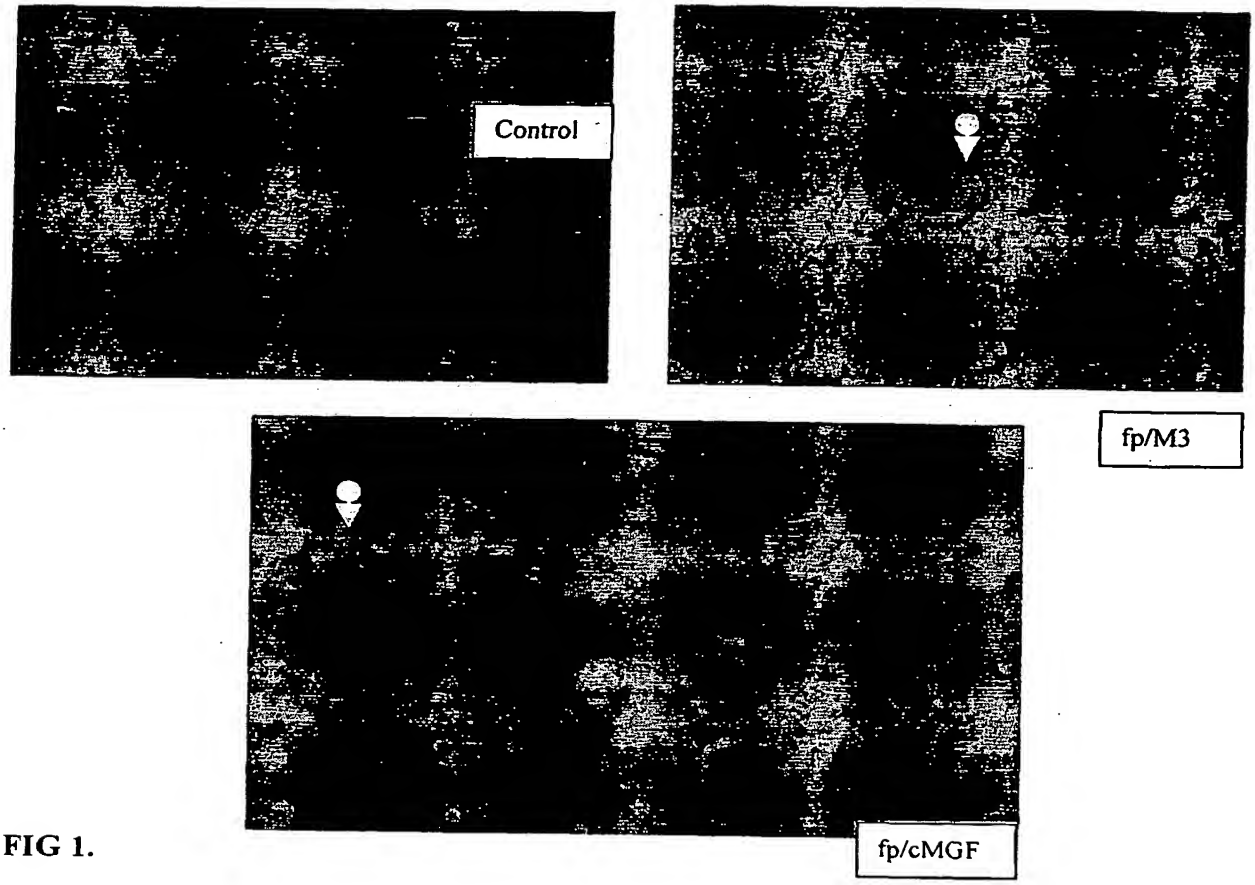

FIG 1.

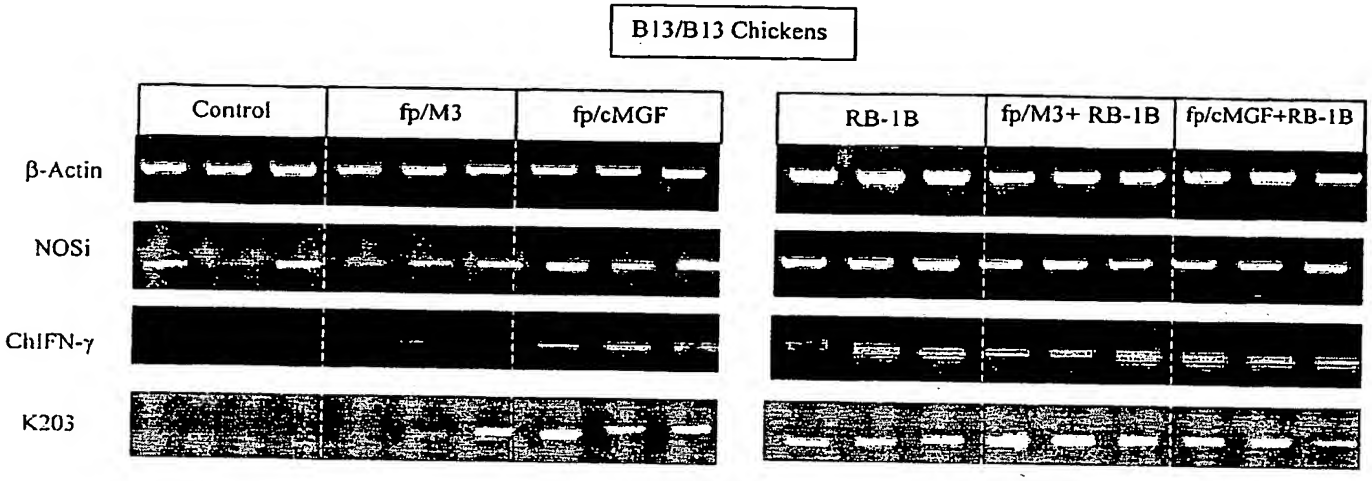
B13/B13 Chickens
Control
fp/M3
fp/cMGF
RB-1B
fp/M3+ RB-1B
fp/cMGF+RB-1B
β-Actin
NOSi
ChIFN-γ
K203

FIG 2.

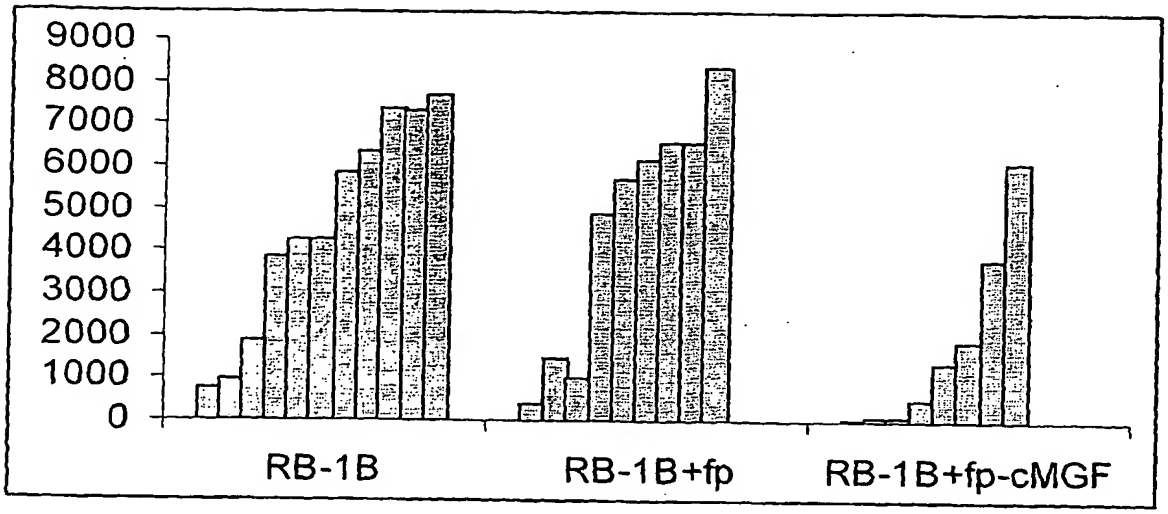

FIG 3.

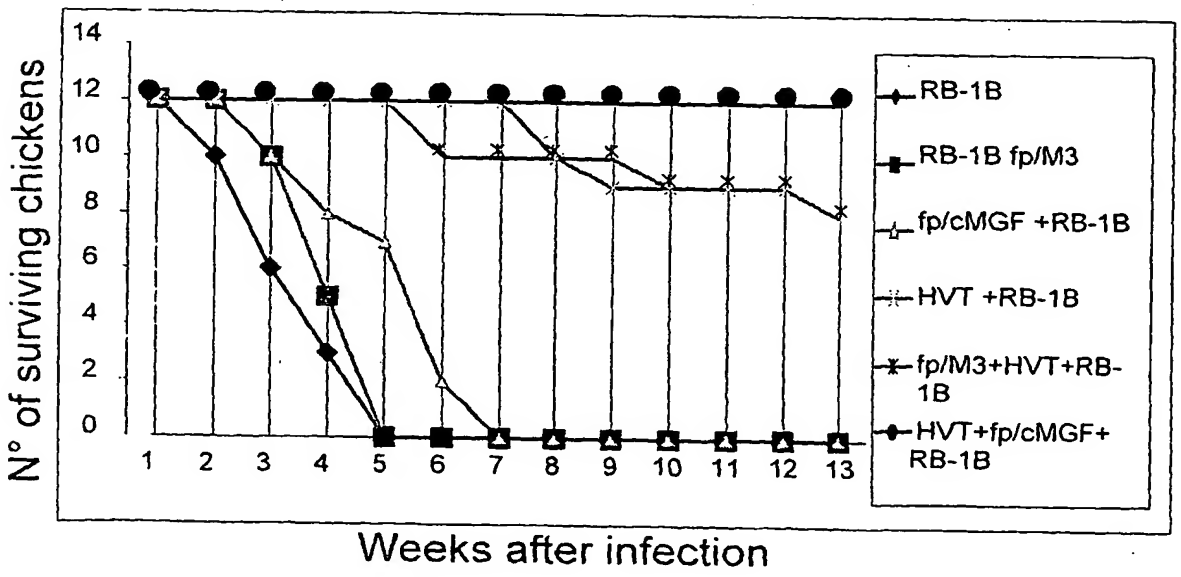

FIG 4.

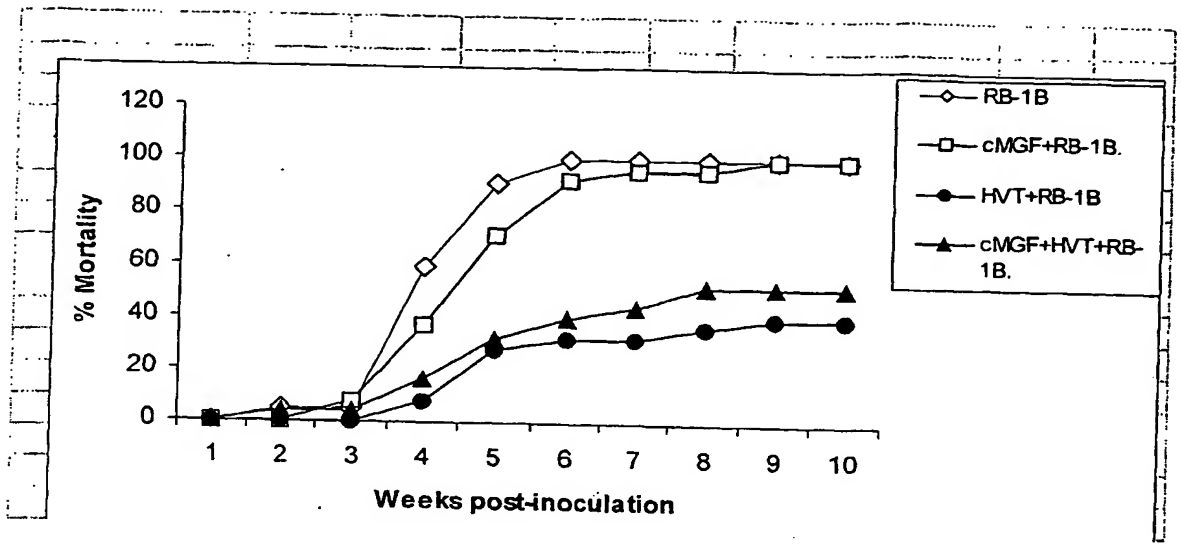
120
100
80
60
40
20
0
% Mortality
1
2
3
4
5
6
7
8
9
10
Weeks post-inoculation
RB-1B
cMGF+RB-1B.
HVT+RB-1B
cMGF+HVT+RB-1B.

FIG 5.

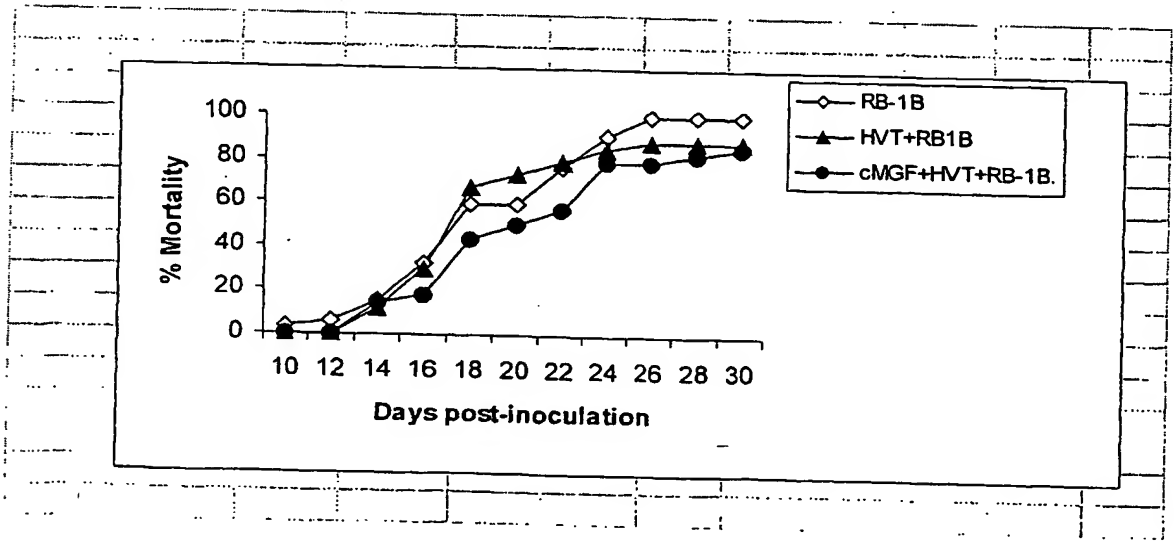
100
80
60
40
20
0
% Mortality
10 12 14 16 18 20 22 24 26 28 30
Days post-inoculation
RB-1B
HVT+RB1B
cMGF+HVT+RB-1B.

FIG 6.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/00800

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 38/18, 38/19, 48/00; A61P 31/12; C12N 15/19, 15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SEE ELECTRONIC DATABASES BELOW.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DERWENT, MEDLINE: Keywords used - mgf, cmgf, myelomonocyte growth factor, chicken, avian, fowl, bird, poultry, turkey, duck, goose, geese

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KARACA, K. et al. Recombinant fowlpox viruses coexpressing chicken type I IFN and Newcastle disease virus HN and F genes: influence of IFN on protective efficacy and humoral responses of chickens following *in ovo* or post-hatch administration of recombinant viruses. Vaccine 1998, Vol.16, No.16, pages 1496-1503. See entire document.	1-72
X	WO A1 96/40880 (SYNTRO CORPORATION) 19 December 1996. See entire document, in particular the examples and claims 9-12.	1-72
X	WO A1 96/27666 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 12 September 1996. See entire document.	1-72

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 2002	14 AUG 2002
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**JULIE CAIRNDUFF** Telephone No : (02) 6283 2545

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/00800

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO A1 96/06170 (EMBREX, INC.) 29 February 1996. See entire document, in particular page 6, lines 2-5 and claims 7-32.	1-8, 11, 16-32, 38, 40-49, 56-64
X	JOHNSON, M.A. et al. Delivery of avian cytokines by adenovirus vectors. Developmental and Comparative Immunology 2000, Vol.24, pages 343-354. See entire document.	1-29, 43-49
X	LILLEHOJ, H.S. and CHOI, K.D. Recombinant chicken interferon-gamma-mediated inhibition of Eimeria tenella development in vitro and reduction of oocyst production and body weight loss following Eimeria acervulina challenge infection. Avian Diseases, 1998, Vol.42, pages 307-314. See entire document.	1-29, 43-49
X	WO AI 00/28003 (THE MACFARLANE BURNET CENTRE FOR MEDICAL RESEARCH LIMITED et al.) 18 May 2000. See entire document, in particular example 2 and claims 1-8.	50-55
X	LEONG, K.H. et al. Selective induction of immune responses by cytokines coexpressed in recombinant fowlpox virus. Journal of Virology, 1994, Vol.68, No.12, pages 8125-8130. See entire document.	50-55

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU02/00800

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	96/40880	AU	64819/96	CA	2223591	EP	832197
WO	96/27666	AU	47792/96	CA	2214453	EP	815233
		NZ	302188	US	6083724		
WO	96/06170	AU	29685/95	CA	2197650	EP	777733
		IL	114685	ZA	9506917		
WO	00/28003	AU	15014/00	EP	1129177		

END OF ANNEX